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# CONGELACIÓN CONVENCIONAL Y ULTRA-RÁPIDA DE ESPERMATOZOIDES DE RUMIANTES SILVESTRES: INFLUENCIA DE LA TESTOSTERONA Y LA PROLACTINA EN LA CRIORRESISTENCIA ESPERMÁTICA

TESIS DOCTORAL

Presentada por:

**Paula Bóveda Gómez**

**Bajo la dirección de los doctores**

Julián Santiago-Moreno

Milagros Cristina Esteso Díez

**Tutora académica**

Joaquina de la Torre Escudero

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# *Mención internacional*

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**Tutor de estancia internacional**

Rodolfo Ungerfeld Morón

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*A mi familia,*

*en especial a mis padres,*

*a mi abuela y a mi hermana*

*“No es la especie más fuerte la que sobrevive,*

*ni la más inteligente,*

*sino la que responde mejor al cambio”*

Charles Darwin

# RESUMEN

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La limitada efectividad en la criopreservación de espermatozoides en especies de rumiantes silvestres determina que se debe hacer un nuevo enfoque de criobiología espermática para conocer los cambios fisiológicos en los espermatozoides durante el ciclo reproductivo anual. Además, también deben desarrollarse métodos alternativos de criopreservación, como la congelación ultra-rápida. Por otro lado, los cambios en el estado endocrino están relacionados con la estacionalidad de estas especies, lo que podría estar afectando a la criopreservación espermática. La presente tesis doctoral ha abordado la criobiología espermática de estas especies desde diferentes perspectivas, con los objetivos de determinar la eficacia de la congelación ultra-rápida de espermatozoides de rumiantes silvestres como alternativa a la congelación convencional, y evaluar la influencia de diferentes cambios hormonales estacionales en la respuesta de los espermatozoides frente a procesos de criopreservación. El primer objetivo específico se desarrolló mediante el artículo I, con el objeto de determinar si mediante la congelación ultra-rápida se consigue un estado vítreo del medio extracelular, y comparar, mediante técnicas convencionales de análisis espermático y de microscopía electrónica de transmisión y de barrido, el daño causado en los espermatozoides mediante dos métodos de criopreservación: congelación ultra-rápida y convencional. La aplicación de estas técnicas de microscopía, sugirió que el estado vitrificado no se alcanzó mediante la congelación ultra-rápida, y provocó más daño en membrana que la congelación lenta convencional, quizás debido a las características de los cristales de hielo formados extracelularmente. El segundo objetivo específico se llevó a cabo mediante el artículo II, para examinar la eficacia de ambos métodos de criopreservación en espermatozoides epididimarios obtenidos *post mortem* mediante una técnica de lavado retrógrado. Los resultados mostraron que, a pesar de ejercer un efecto más dañino sobre las variables espermáticas que la congelación convencional, la congelación ultra-rápida puede ser una alternativa útil para la criopreservación de espermatozoides epididimarios para estas especies en condiciones de campo. El tercer objetivo específico se desarrolló mediante el artículo III, para examinar la influencia de los cambios de secreción de testosterona en la criorresistencia espermática, utilizando ambos métodos de criopreservación; en un primer experimento se redujeron los niveles de testosterona usando el anti-andrógeno acetato de ciproterona (CA), y en un segundo experimento los niveles de testosterona se incrementaron mediante la administración de propionato de testosterona (TP). Ambos tratamientos modificaron las concentraciones plasmáticas de testosterona como se esperaba, y los resultados mostraron que las concentraciones circulantes de testosterona parecen influir en la criorresistencia de los espermatozoides. Por último, el cuarto objetivo específico se llevó a cabo a través del artículo IV, con el fin de examinar la influencia de los cambios inducidos en la secreción de prolactina (PRL), en la criorresistencia espermática mediante los mismos métodos de criopreservación; en un primer experimento la secreción PRL fue modificada usando el agonista dopaminérgico bromocriptina (BCR), y en un segundo experimento usando el antagonista domaminérgico D2 sulpirida (SLP). Ambos tratamientos modificaron las concentraciones plasmáticas de PRL y los resultados sugirieron que los altos

niveles de PRL afectan negativamente la criorresistencia de los espermatozoides. Los resultados obtenidos en la presente tesis han permitido incrementar los conocimientos sobre criobiología espermática y fisiología reproductiva en los rumiantes silvestres, lo que permitirá la optimización del desarrollo de biotecnologías reproductivas en estas especies.

# ABSTRACT

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The limited effectiveness on sperm cryopreservation in wild ruminant species determines that a new sperm cryobiology approach should be made to know physiological changes in sperm during the annual reproductive cycle. Moreover, alternative cryopreservation methods, such as ultra-rapid frozen, should also be developed. In turn, changes in endocrine status are related with the seasonality in these species, and a possible influence could be affecting the sperm cryopreservation. The aims of the present doctoral thesis were to determine the effectiveness of ultra-rapid freezing in sperm of wild ruminants like alternative to conventional freezing, and to evaluate the influence of different seasonal hormonal changes on the sperm response to cryopreservation process. In the first article the physical state of the extracellular milieu was examined to determine whether ultra-rapid cooling vitrifies the extracellular milieu; the damage to sperm caused by two cryopreservation methods (ultra-rapid and conventional) was compared using conventional sperm analysis techniques and scanning and transmission electron microscopy. The application of these microscopy techniques suggested that the vitrified state was not achieved by ultra-rapid cooling and provoked more membrane damage than slow cooling, perhaps due to the characteristics extracellular ice crystals formed. The second specific aim was developed by the article II, to examine the effectiveness of both cryopreservation methods for *post-mortem* epididymal sperm obtained by retrograde flushing technique. Results showed that despite exerting more harmful effect on sperm variables than conventional freezing, ultra-rapid freezing may be a useful alternative for the cryopreservation of epididymal sperm for these species in field studies. The third specific aim was developed by the article III, to examine the influence of induced changes in testosterone secretion on sperm cryoresistance by both cryopreservation methods in samples collected *in vivo*; in a first experiment testosterone levels were reduced using the antiandrogen cyproterone acetate (CA), and in a second experiment testosterone levels were increased using the androgen testosterone propionate (TP). Both treatments modified the plasma testosterone concentration as expected and results showed that the circulating testosterone concentrations appear to influence sperm cryoresistance. Finally, the fourth specific aim was developed by the article IV, to examine the influence of induced changes in prolactin (PRL) secretion on sperm cryoresistance by ultra-rapid and conventional cryopreservation method; in a first experiment PRL secretion was modified using dopamine agonist bromocriptine (BCR), and in a second experiment dopamine $D_2$-receptor antagonist sulpiride (SLP) was used. Both treatments modified the plasma PRL concentrations and results suggested that high levels of PRL negatively affect the sperm cryoresistance. The evidences obtained have increased the knowledge on sperm cryobiology and on the reproductive physiology of wild ruminants, which could facilitate the optimization and the improvement of the reproductive biotechnologies in these species.

# OBJETIVOS

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## Objetivos generales

Determinar la eficacia la congelación ultra-rápida de espermatozoides de rumiantes silvestres, como alternativa a la congelación convencional, y evaluar cómo actúan los cambios hormonales estacionales en la respuesta de los espermatozoides a los procesos de criopreservación.

## Objetivos específicos:

**1)** Estudio comparativo de los daños celulares (estructurales y funcionales) entre congelación lenta convencional y congelación ultra-rápida. Evaluación de posibles procesos de vitrificación intra- y extracelulares.

**2)** Evaluar la eficacia de la congelación ultra-rápida en espermatozoides epididimarios obtenidos *post-mortem*.

**3)** Influencia de la testosterona en la respuesta a la criopreservación (congelación convencional y ultra-rápida).

**4)** Influencia de la prolactina en la respuesta a la criopreservación (congelación convencional y ultra-rápida).

# INTRODUCCIÓN

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Los avances en las biotecnologías reproductivas en especies silvestres han estado limitados por las dificultades en su accesibilidad y manejo. Sin embargo, en los últimos años hemos asistido al desarrollo de nuevas técnicas de criopreservación espermática en especies silvestres, en muchos casos como extrapolación de modelos en especies domésticas, que ha favorecido el desarrollo de bancos de recursos genéticos, la optimización de explotaciones cinegéticas para la mejora genética a partir de ejemplares de valor singular, y significativos avances en la conservación de especies silvestres amenazadas. Un requisito necesario para garantizar el éxito de todos estos avances en la aplicación de tecnologías reproductivas como la criopreservación espermática, es incrementar el conocimiento de los daños espermáticos en función de las rampas de congelación empleadas, así como de la fisiología reproductiva de cada una de las especies, y evaluar de qué manera sus cambios estacionales reproductivos pueden afectar a la efectividad de estas técnicas. En el caso concreto de los ungulados silvestres en España, todo esto ha sido revelador para determinadas especies como es el caso del muflón (*Ovis musimon*), ciervo rojo (*Cervus elaphus*), gamo (*Dama dama*) y cabra montés (*Capra pyrenaica*), en las que se ha podido caracterizar diferentes parámetros reproductivos, tales como la estacionalidad reproductiva o el ciclo sexual, gracias a los avances en nuevos métodos de monitorización de la actividad reproductiva (Santiago-Moreno y López-Sebastián, 2010). Una mejor comprensión del posible papel del estatus endocrino y sus fluctuaciones, a lo largo del año, en la criorresistencia espermática durante los procesos de criopreservación optimizaría la implementación de bancos de recursos genéticos y el desarrollo de diferentes biotecnologías reproductivas.

## 1. Criopreservación espermática

Todo proceso de criopreservación implica importantes daños a nivel físico-químico en las estructuras de la célula espermática debido a los cambios osmóticos y de temperatura que se producen a lo largo de las distintas etapas de enfriamiento (Morris et al., 2012).

La congelación convencional con ramplas de enfriamiento lenta implica un tiempo de equilibrado a 5°C de temperatura, que varía en función de la especie y el origen de la muestra espermática, seguido de una congelación mediante vapores de nitrógeno durante 10 minutos (Garde et al., 2003; Pradiee et al., 2016). Junto a ello, la adición de yema de huevo como aditivo en estos medios de congelación tiene unos resultados beneficiosos gracias a su acción de protección sobre las células espermáticas durante la congelación y descongelación (Aboagla y Terada, 2004).

A pesar de los recientes avances en la congelación clásica, los medios de congelación implicados para llevarla a cabo no evitan la formación de cristales a nivel extra e intracelular. Estos cristales implican un daño sobre las células y afectan de manera directa a su criorresistencia. Como alternativa, recientemente, diferentes estudios abordaron la posibilidad de conseguir la vitrificación espermática mediante uso de rampas de enfriamiento ultra-rápidas que se conseguían mediante la inmersión de pequeños volúmenes de muestra directamente sobre nitrógeno líquido (Isachencko et al., 2003). El uso de rampas de enfriamiento ultra-rápidas, debería prevenir la formación de cristales de hielo en el interior de las células, favoreciendo el establecimiento de un medio vítreo extra- e intra-celular. Además, estas técnicas son menos costosas y fáciles de aplicar en condiciones de campo, lo que podría suponer una alternativa de gran valor en la criopreservación de material espermático en especies silvestres. El uso de esta técnica implica la adición de concentraciones altas de agentes crioprotectores que suponen una mayor toxicidad sobre las células espermáticas (Isachenko et al., 2003). Aun así, se han conseguido optimizar protocolos para intentar aplicar este método como una alternativa al convencional, con concentraciones moderadas de crioprotectores no penetrantes como la sucrosa, que permite someter a las células a rampas de enfriamiento ultra-rápidas, pero con una metodología mucho más sencilla que la congelación convencional, lo que puede llegar a ser muy práctico cuando se trabaja con especies silvestres en condiciones de campo (Pradiee et al., 2015; 2017).

Los daños que se producen en las células espermáticas, durante el proceso de criopreservación, en muchos casos llegan a ser irreversibles. La reducción de los daños por shock térmico, osmótico y cristales de hielo a nivel intracelular es una de las claves para conseguir una mejor eficiencia en la criopreservación de las células espermáticas por lo que, el estudio sobre crioprotectores y otros aditivos, junto a la optimización de rampas de enfriamiento es fundamental (Drobnis, 1993). Por ello, la composición del medio en el que las células se congelan debe mantener unas condiciones adecuadas de pH y osmolaridad para favorecer la máxima supervivencia espermática frente a cambios de temperatura durante los procesos de refrigeración y de congelación-descongelación (Holt, 2000).

Para optimizar protocolos de criopreservación espermática hay que tener muy en cuenta las concentraciones de los agentes crioprotectores, ya que pueden llegar a ser tóxicas para la célula, afectando negativamente sobre su viabilidad (Gao et al., 1993). El descubrimiento del glicerol como crioprotector, marcó un avance en la criopreservación del semen y su uso se ha puesto a punto en la mayoría de los protocolos de congelación actuales. Este crioprotector, combinado con otros aditivos es el más usado para la criopreservación lenta o convencional en la mayoría de las especies, siendo el más usado en pequeños rumiantes para programas de conservación de especies (Curry 2000).

El estudio de los daños producidos a nivel celular es muy importante para poder entender y optimizar mejor cualquier protocolo de congelación. A raíz de ello, se ha planteado un primer objetivo específico para el estudio de las alteraciones ultraestructurales y funcionales que pueden acontecer durante los dos

procesos de congelación-descongelación. Además, se ha investigado si realmente se alcanza un estado vítreo extracelular durante la congelación ultra-rápida. Con este objetivo se ha llevado a cabo la redacción del primer artículo dentro de esta tesis.

## 2. Influencia del origen espermático (epididimario y eyaculado) en la criorresistencia espermática

La criopreservación de espermatozoides en especies silvestres está muy limitada debido a las condiciones de trabajo en campo, lo que dificulta la obtención de las muestras y la congelación de las mismas. En muchos casos, la obtención de material espermático *post-mortem* es la única opción para tener acceso a este tipo de muestras biológicas. La criorresistencia de los espermatozoides puede estar asociado al origen espermático: epididimario *post-mortem* y eyaculado. El plasma seminal y los fluidos epididimarios presentan composiciones diferentes que pueden afectar a la criorresistencia y al estado de capacitación de las muestras espermáticas, según sean eyaculadas o epididimarias, por lo que el método de obtención de la muestra seminal afecta a la sensibilidad del espermatozoide a los procesos de criopreservación, debido a las variaciones que se producen en la composición de la membrana plasmática y en el proteoma del espermatozoide (Jiménez-Ramadán et al., 2016; Martínez-Fresneda et al., 2021). De hecho, se ha apuntado una mayor criorresistencia en muestras espermáticas epididimarias (Martínez-Fresneda et al., 2019, 2021).

La limitada efectividad de la criopreservación en estas especies implica nuevos planteamientos dentro del campo de la criobiología espermática, como es la adaptación de protocolos en función del método de obtención y especie, debido a las limitaciones encontradas en condiciones de trabajos de campo. Esto nos ha llevado a plantear el segundo objetivo de esta tesis doctoral, que ha sido evaluar la eficacia de dos métodos de congelación (clásica y ultra-rápida) en espermatozoides de origen epididimario obtenidos *post-mortem*. Para este objetivo se han utilizado espermatozoides epididimarios por presentar mayor criorresistencia en comparación con espermatozoides obtenidos de muestras eyaculadas. Esto ha permitido desarrollar el segundo artículo dentro de esta tesis doctoral.

## 3. Influencia del estatus endocrino en la criorresistencia espermática

Las marcadas variaciones estacionales de la actividad reproductiva y fisiología espermática en las distintas especies de rumiantes silvestres determinan variaciones en los procedimientos utilizados para la criopreservación espermática (Santiago-Moreno et al., 2006a; Pradiee et al., 2016). Un perfecto

conocimiento de la estacionalidad reproductiva y de las variaciones endocrinas que acontecen a lo largo del año, supone un pilar de conocimiento fundamental para abordar el estudio de papel del estatus endocrino en la congelabilidad de los espermatozoides.

### 3.1. Estacionalidad reproductiva y congelabilidad de la célula espermática

Las diferentes especies de ungulados silvestres mantenidas en su hábitat natural están sometidas a las variaciones de horas luz/día a lo largo del año (fotoperiodo), que depende de su situación geográfica (latitud), la temperatura y las interacciones sociales, entre otros. El fotoperiodo es el principal factor medioambiental que regula la estacionalidad reproductiva. La adaptación genética a las distintas condiciones medioambientales donde se desarrolla una población, determina el desarrollo de diferentes estrategias reproductivas adaptativas (Bronson, 1989). El marcado carácter estacional de las especies de rumiantes silvestres se caracteriza por un periodo concreto de actividad reproductiva y otro de reposo sexual (anestro) que varía en función de la especie, las condiciones medioambientales y la localización geográfica de la misma (Heideman y Bronson, 1994). Estos periodos de actividad sexual suelen ser cortos, y las interacciones sociales tienen una gran influencia en las variaciones de la estacionalidad. El inicio del incremento de la actividad testicular precede, en todos los casos, al inicio de la actividad ovulatoria de las hembras (Figura 1).

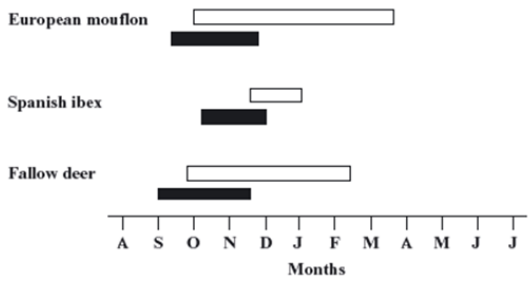


**Figura 1.** Las barras negras representan el máximo periodo de actividad testicular en machos y las barras blancas representan el periodo de actividad ovulatoria en hembras (Imagen tomada de Santiago-Moreno et al., 2006b).

Los cambios que se producen a nivel del sistema reproductivo a lo largo del año están determinados por la sincronización del ritmo endógeno de actividad neuroendocrino-gonadal, a través del fotoperiodo (Lincoln and Short, 1980; Santiago-Moreno et al., 2012). En este proceso, la melatonina actúa como transductor de la información fotoperiódica en una respuesta endocrino–gonadal (Karsch et al., 1984; Lincoln and Clarke, 1995). Es importante tener en cuenta que factores como situaciones de estrés, tales como la cautividad, inmovilización y captura, o estatus jerárquico, influyen sobre este eje neuro-endocrino-gonadal (Marco et al., 1998; Santiago-Moreno et al., 2006b).

A nivel testicular, la LH actúa sobre las células de Leydig, regulando la producción de testosterona (Setchell et al., 1994), mientras que la FSH controla la actividad de las células de Sertoli, regulando la

espermatogénesis (Gerlach y Aurich, 2000). La secreción de testosterona va a modular el correcto desarrollo de las glándulas sexuales accesorias, en función de su patrón de secreción estacional, presentando sus máximos niveles durante el periodo reproductivo (Lincoln, 1998; Santiago-Moreno et al., 2012). A su vez, las variaciones anuales del fotoperiodo también están directamente relacionadas con el ritmo anual de secreción de la prolactina, una hormona secretada principalmente por la hipófisis (Lincoln y Clarke, 1995) y que, en condiciones de fotoperiodo natural, tanto en machos como en hembras, muestra un perfil de secreción estacional, con altas concentraciones en primavera y verano y niveles basales en otoño e invierno. Las concentraciones plasmáticas más altas de prolactina se observan durante los meses de verano (Santiago-Moreno et al., 2005), mientras que las concentraciones basales se alcanzan durante los meses de invierno, coincidiendo con el periodo reproductivo y niveles altos de testosterona (Lincoln, 1998).

En ungulados silvestres, los cambios en el estatus endocrino, dependientes de la estacionalidad reproductiva, plantean su posible influencia sobre los rendimientos de la congelación espermática, ya sea a través de variaciones hormonales en la propia célula, o bien por modificación de otros escenarios, como secreciones a nivel de glándulas sexuales accesorias o a nivel de epidídimo, que afectan a la composición del plasma seminal. Además, estos cambios hormonales pueden tener una relación directa sobre la espermatogénesis.

Como ya se ha mencionado anteriormente, la testosterona es la principal hormona que regula la espermatogénesis. Además, durante el tiempo en el que los espermatozoides permanecen en el tracto reproductivo masculino, están expuestos a variaciones estacionales en los niveles de testosterona. Esta hormona afecta al metabolismo de la célula al igual que tiene una influencia en su potencial de membrana y el transporte de líquidos a través de ella (Calzada et al., 1988). Además, favorece la función de las glándulas sexuales accesorias, que presentan una variación anual de actividad secretora similar al tamaño testicular (Xu et al., 1991; Santiago-Moreno et al., 2006b). Por tanto, los espermatozoides se encuentran expuestos a concentraciones variables de esta hormona a lo largo del año, las cuales pueden influir sobre las propiedades del espermatozoide y su criorresistencia frente a procesos de congelación según la época del año (Purohit et al., 2000).

En el caso de la prolactina, se ha reportado que estimula y regula tanto la función esteroidogénica, como la espermatogénica en el testículo (Fujimoto et al., 2002) y, a su vez, está implicada en la maduración del espermatozoide en el epidídimo (Reddy et al., 1985). Su papel en la función reproductiva del macho es manifiesta al haberse apuntado la existencia de receptores para esta hormona en las glándulas sexuales accesorias, en las células intersticiales de Leydig y en los espermatocitos y espermátidas (Pujianto et al., 2010). Por tanto, la influencia de esta hormona en una mayor o menor sensibilidad a los procesos de congelación de las células espermáticas podría venir derivado de una acción directa sobre el propio espermatozoide o bien, junto a la testosterona, a través de los cambios que

se producen en la secreción de las glándulas sexuales accesorias dependientes de ambas hormonas, o a nivel del epidídimo y el conducto deferente, incluida la ampolla de Henle. A pesar de su importancia en la biología reproductiva de los rumiantes, hasta ahora no se han planteado estudios en los que se hayan determinado sus concentraciones en plasma seminal, su relación con la actividad de las glándulas sexuales accesorias y su posible influencia en la congelabilidad de los espermatozoides de los rumiantes silvestres.

Las ventajas de utilizar modelos de especies silvestres en estudios de fisiología y tecnologías reproductivas de pequeños rumiantes ya han sido puestas de manifiesto previamente en otros trabajos relacionados con el control del fotoperiodo de la actividad neuroendocrinogonadal de los ovinos y caprinos domésticos y silvestres (Santiago-Moreno y López-Sebastián, 2010), y con el papel modulador de la testosterona en la actividad de las glándulas accesorias sexuales y los caracteres sexuales secundarios (Santiago Moreno et al., 2005; Toledano-Díaz et al., 2012). Todos estos estudios ponen de relieve la existencia de fuertes cambios estacionales en la secreción endocrina, y conducen al planteamiento de la hipótesis de variaciones en la respuesta de las células espermáticas a los procesos de congelación, en función del estatus endocrino en el que se encuentre.

Estudios previos han observado que el periodo que ofrece los mejores resultados frente a los procesos de congelación/descongelación, en términos de motilidad, viabilidad, integridad del acrosoma y funcionalidad de membrana plasmática, no es, como podría esperarse, aquel que coincide con las mayores concentraciones de testosterona y óptima calidad espermática, al inicio de la estación sexual, sino que es al final de la misma. Además, el periodo que ofrece los mejores resultados de congelabilidad espermática coincide con la disminución considerable de los niveles de testosterona, si bien los espermatozoides derivan de un ciclo espermatogénico previo con óptimas condiciones de niveles de andrógenos (Coloma et al., 2010). Los dos últimos objetivos específicos de esta tesis abordan la cuestión del posible papel del estatus endocrino en las variaciones anuales de la criorresistencia espermática, centrándose en las hormonas testosterona y prolactina.

# MATERIAL Y MÉTODOS

# MATERIAL Y MÉTODOS

## 1. Generalidades y biología de las especies modelo de estudio

### *1.1. Cabra montés (Capra pyrenaica;* Schinz 1838*)*

Se caracteriza por un marcado ciclo anual de actividad testicular similar al de otras especies de ungulados (Lincoln y Davidson, 1977; Asher et al., 1999), con el aumento del tamaño de los testículos y de la secreción de testosterona unos meses antes del inicio de la época reproductiva (septiembre-octubre). Con el incremento de la secreción de testosterona se empiezan a producirse cambios a nivel de comportamiento (agresividad y luchas intrasexuales) y optimización de la actividad espermatogénica (Figura 2). El pico de secreción de testosterona se produce en los mese de octubre y noviembre. El periodo reproductivo en esta especie está definido en los meses de diciembre-enero.

**Figura 2.** Ejemplares de macho montés en su espacio natural.

### *1.2. Muflón (Ovis musimon;* Pallas, 1762*)*

Presenta una estacionalidad muy marcada en su actividad reproductiva, la cual se refleja en la evolución anual de las variaciones en las concentraciones plasmáticas de testosterona, junto a variaciones en el tamaño testicular (Lincoln, 1998; Santiago-Moreno et al., 2005). El incremento de las concentraciones de testosterona comienza en el mes de septiembre, y se acompañan del inicio del comportamiento agresivo. La máxima actividad testicular acontece entre los meses de octubre a diciembre (Figura 3). El periodo reproductivo en esta especie está definido en los meses de octubre-abril.

**Figura 3.** Ejemplares macho de muflón en condiciones de cautividad.

### *1.3. Gamo (Dama dama;* Linnaeus, 1758*)*

La estacionalidad de la actividad reproductiva en el macho se ve reflejada en las variaciones anuales en el tamaño testicular y en la secreción de testosterona, los cuales se están directamente relacionados con los cambios morfológicos que se producen en el crecimiento y desmogue de la cuerna. A diferencia de las dos especies anteriores su actividad espermatogénica cesa completamente fuera de la estación reproductiva, llegando a tener muestras seminales azoospérmicas durante un corto periodo de tiempo (mayo-junio) en el que el tamaño testicular alcanza valores mínimos (Gosch y Fischer, 1989) (Figura 4). El incremento de la actividad testicular en septiembre, inicio de la ronca, precede al inicio de la actividad ovulatoria de las gamas que se inicia en el mes de octubre y finaliza en abril (Santiago-Moreno y López Sebastián, 2010).

**Figura 4.** Ejemplares macho y hembra de gamo en condiciones de cautividad.

## 2. Manejo y obtención de muestras seminales en ungulados silvestres

### *2.1. Condiciones de parques y captura de animales*

En esta tesis doctoral, el manejo de los animales *in vivo* se ha realizado mediante captura física (capturaderos) y captura química, esta última mediante el uso de fármacos anestésicos administrados en el mismo capturadero una vez inmovilizado el animal (Figuras 1-3, anexo). La adecuada selección de los fármacos anestésicos es importante a la hora de obtener la muestra espermática en estas especies, ya que su uso puede interferir con mecanismos neuromusculares que controlan las funciones de erección y eyaculación. Además, cuando se trabaja con rumiantes silvestres, es importante tener en cuenta toda complicación que se pueda producir tras la captura química, por lo que una adecuada reversión de la anestesia es fundamental para los animales (Santiago-Moreno et al., 2011) (Figura 4, anexo).

### *2.2. Técnicas de monitorización y mantenimiento*

En las especies estudiadas una vez inducida la anestesia, se realizaba el mantenimiento mediante anestesia inhalatoria junto a una monitorización del animal para tener un control en todo momento de sus variables de temperatura, frecuencia cardiaca, respiratoria y saturación de oxígeno y $CO_2$ (Figura 5, anexo). El estudio ecográfico ha proporcionado un estudio detallado de las variaciones estacionales del tamaño testicular y de las glándulas sexuales accesorias durante la obtención de muestras seminales (Figuras 6 y 7, anexo).

### *2.3. Métodos de obtención de muestras espermáticas*

- *Recogida post-mortem mediante lavado retrógrado de epidídimo*. Este lavado se ha realizado de manera independiente en cada uno de los testículos por separado, utilizando para el mismo el diluyente correspondiente en función del método de criopreservación utilizado tras la recogida (Figura 8, anexo) (Pradiee et al., 2014).
- *Recogida in vivo mediante método masaje transrectal guiado con ultrasonido (TUMASG).* Esta técnica se ha llevado a cabo durante toda la recogida de muestras espermáticas necesaria para la evaluación de la influencia del estatus endocrino (Figura 9, anexo) (Santiago-Moreno et al., 2013).

## 3. Técnicas de evaluación y procesado de muestras espermáticas

### *3.1. Evaluación de parámetros espermáticos*

- *Concentración espermática.* Mediante microscopía óptica de contraste de fases (40x).
- *Motilidad*. Mediante sistema de análisis computerizado de imágenes (CASA; SCA) (Figura 10, anexo) (Santiago-Moreno et al., 2013).

- *Viabilidad celular e integridad del acrosoma junto a evaluación del estado de la membrana plasmática, membrana del acrosoma y membrana mitocondrial*. Mediante microscopía óptica de fluorescencia y el uso de una combinación de fluorocromos para la evaluación simultánea de la viabilidad (yoduro de propidio; PI) y de la integridad del acrosoma (aglutinina de *Arachis hypogaea* - PNA, marcada con el fluorocromo FITC) (Figura 11, anexo) (Soler et al., 2005). A su vez, mediante el uso de Mitotracker Green para evaluar el estado de membranas (Forero-González et al., 2012).
- *Viabilidad mediante técnicas de tinción eosina/nigrosina.* Mediante microscopía de campo claro (Figura 11, anexo) (Campbell et al., 1956).
- *Morfología espermática*. Mediante evaluación de microscopía óptica de contraste de fases, determinando los tipos de morfoanomalías (Figura 11, anexo) (Frank 1950).
- *Test de integridad de acrosoma (NAR) y test de endósmosis para la integridad de membrana (HOST).* Mediante evaluación de microscopía óptica de contraste de fases (Figura 11, anexo) (Jeyendran et al., 1984; Pintado y Pérez-Llano, 1992).
- *Morfometría espermática*. Mediante la técnica de tinción hemacolor y programa SCA El sistema ASMA (Módulo de morfología dentro del SCA), ha permitido obtener distintos parámetros de medida para la cabeza de los espermatozoides (Figura 12, anexo) (Esteso et al., 2015).

### *3.2. Criopreservación espermática*

- *Método de congelación lenta o convencional*. En función del origen de la muestra, el tiempo de equilibrado ha sido diferente: en muestras obtenidas *post-mortem* ha sido de 1 hora de equilibrado y refrigeración a 5º C, mientras que en muestras obtenidas *in vivo* ha sido de un total de 3 horas a 5º C (1 hora de refrigerado junto a 2 horas de mantenimiento a esa misma temperatura). La congelación se ha realizado mediante pajuelas de 0,25 ml mediante vapores de nitrógeno líquido durante 10 minutos (Figura 13, anexo) (Pradiee et al., 2014).
- *Método de congelación ultra-rápida*. En este caso, el tiempo de equilibrado ha sido de 30 minutos a 5º C independientemente del origen de la muestra. La congelación se ha realizado mediante pellets de 50 µl, sumergidos directamente sobre el nitrógeno líquido (Figura 14, anexo) (Pradiee et al., 2015).

### *3.3. Descongelación de muestras espermáticas*

- *Método de congelación lenta o convencional.* Las muestras han sido descongeladas en un baño maría durante 30 segundos a 37º C (Pradiee et al., 2014, 2015).
- *Método de congelación ultra-rápida.* Las muestras han sido descongeladas de manera ultra-rápida mediante el DPP70® (INIA), compuesto por placas termorreguladas a una temperatura entre los 60-65º C (Figura 14, anexo) Pradiee et a., 2015).

### *3.4. Evaluación mediante técnicas de microscopía electrónica*

- *Crio-microscopía electrónica de barrido (Crio-SEM)*. Esta técnica se ha usado para determinar el estado del medio extracelular dependiendo del método de congelación en el cual se han congelado las células espermáticas (Figura 15, anexo) (Bóveda et al., 2020).
- *Microscopía electrónica de barrido (SEM).* Esta técnica se ha usado para determinar los daños producidos a nivel celular en las células espermáticas, tras haber sido sometidas a ambos procesos de congelación (Figura 15, anexo) (Bóveda et al., 2020).
- *Microscopía electrónica de transmisión (TEM).* Esta técnica se ha usado para determinar los daños producidos a nivel de las organelas de las células espermáticas, tras haber sido sometidas a ambos procesos de congelación (Figura 15, anexo) (Bóveda et al., 2020).

### *3.5. Evaluación de concentraciones de testosterona y prolactina mediante técnicas de radioinmunoanálisis (RIA)*

Esta técnica se ha utilizado para determinar las concentraciones hormonales de testosterona y prolactina de las muestras congeladas, tanto en plasma sanguíneo como en plasma seminal, obtenidas durante toda la fase de recogida de muestras realizada en base a los diseños experimentales dentro de esta tesis (Santiago-Moreno et al., 2005).

# *ARTÍCULO I*

## Ultra-rapid cooling of ibex sperm by spheres method does not induce a vitreous extracellular state and increases the membrane damages

*PLoS ONE* 15(1): e0227946 (Bóveda et al., 2020)

## La congelación ultra-rápida mediante el método de esferas no induce un estado vítreo extracelular e incrementa el daño de las membranas

## RESUMEN ARTÍCULO I

La criopreservación espermática mediante congelación ultra-rápida, basada en la disposición de pequeños volúmenes de muestras espermáticas diluidas directamente sobre el nitrógeno líquido, ha tenido éxito en algunas especies de rumiantes silvestres, incluyendo la cabra montés (*Capra pyrenaica*). Es esperable que la criopreservación ultra-rápida determine un estado vítreo extracelular, pero no existen estudios que hayan investigado de forma objetiva la presencia o ausencia de formación de hielo en el medio extracelular cuando se aplica esta técnica. Las distintas modificaciones que se produzcan en el medio extracelular probablemente ejerzan algún tipo de daño en el plasmalema y en membranas del acrosoma y mitocondrias. Los objetivos del presente trabajo han sido: 1) examinar el estado físico el medio extracelular durante una criopreservación a velocidades de enfriamiento lentas y ultra-rápidas—para determinar si las rampas de enfriamiento ultra-rápidas vitrificas el medio extracelular; y 2) comparar, utilizando técnicas convencionales de análisis espermático y microscopía electrónica de transmisión y de barrido, el daño en los espermatozoides causado por ambos métodos de criopreservación. Las muestras espermáticas fueron obtenidas mediante el método de masaje transrectal guiado por ultrasonido (TUMASG) en machos monteses anestesiados, y criopreservadas usando técnicas de congelación lenta y ultra-rápida. La motilidad espermática (22,95 ± 3,22% vs 4,42 ± 0,86%), viabilidad (25,64 ± 3,71% vs 12,8 ± 2,50%), integridad del acrosoma (41,45± 3,73% vs 27,00 ± 1,84%) e integridad de membrana mitocondrial (16,52 ± 3,75% vs 4,00 ± 0,65%) fueron mejores después de la congelación lenta ($P<0.001$), que tras la congelación ultra-rápida. La criomicroscopía electrónica de barrido (Cryo-SEM) ha determinado que no se llega a alcanzar un estado vítreo mediante la congelación ultra-rápida, y que los cristales de hielo formados han sido más pequeños y más estriados ($P<0.001$) que mediante la congelación lenta. La microscopía electrónica de barrido no reveló diferencias en los tipos de daño celular entre ambas técnicas de criopreservación. Por otro lado, la microscopía electrónica de transmisión mostró daños más intensos a nivel en plasmalema y membrana mitocondrial con la congelación ultra-rápida. En conclusión, la congelación ultra-rápida ha provocado más daño a nivel de membrana que la congelación lenta, probablemente debido a las características de los hielos formados en el medio extracelular.

PLOS ONE

RESEARCH ARTICLE

# Ultra-rapid cooling of ibex sperm by spheres method does not induce a vitreous extracellular state and increases the membrane damages

**Paula Bóveda[1], Adolfo Toledano-Díaz[1], Cristina Castaño[1], Milagros Cristina Esteso[1], Antonio López-Sebastián[1], Dimitrios Rizos[1], Alejandro Bielli[2], Rodolfo Ungerfeld[3], Julián Santiago-Moreno[1]***

**1** Dpto. Reproducción Animal, INIA, Madrid, Spain, **2** Dpto. Morfología y Desarrollo, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay, **3** Dpto. Fisiología, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay

* moreno@inia.es





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## Abstract

Sperm cryopreservation by ultra-rapid cooling based on dropping small volumes of sperm suspension directly into liquid nitrogen, has been successful in some wild ruminant species, including the Iberian ibex (*Capra pyrenaica*). In ultra-rapid cooling, the contents of these droplets are expected to enter a stable, glass-like state, but to the best of our knowledge no information exists regarding the presence or absence of ice formation in the extracellular milieu when using this technique. Different modifications to the extracellular milieu likely inflict different types of damage on the plasmalemma, the acrosome and mitochondrial membranes. The aims of the present work were: 1) to examine the physical state of the extracellular milieu after cryopreservation at slow and ultra-rapid cooling rates—and thus determine whether ultra-rapid cooling vitrifies the extracellular milieu; and 2) to compare, using conventional sperm analysis techniques and scanning and transmission electron microscopy, the damage to sperm caused by these two methods. Sperm samples were obtained by the transrectal ultrasound-guided massage method (TUMASG) from anesthetized Iberian ibexes, and cryopreserved using slow and ultra-rapid cooling techniques. Sperm motility (22.95 ± 3.22% vs 4.42 ± 0.86%), viability (25.64 ± 3.71% vs 12.8 ± 2.50%), acrosome integrity (41.45± 3.73% vs 27.00 ± 1.84%) and mitochondrial membrane integrity (16.52 ± 3.75% vs 4.00 ± 0.65%) were better after slow cooling (P<0.001) than after ultra-rapid technique. Cryo-scanning electron microscopy (Cryo-SEM) suggested that the vitrified state was not achieved by ultra-rapid cooling, and that the ice crystals formed were smaller and had more stretchmarks (P<0.001) than after slow cooling. Scanning electron microscopy revealed no differences in the types of damage caused by the examined techniques, although transmission electron microscopy showed the damage to the plasmalemma and mitochondrial membrane to be worse after ultra-rapid cooling. In conclusion ultra-rapid cooling provoked more membrane damage than slow cooling, perhaps due to the extracellular ice crystals formed.

## Introduction

Much effort has been invested in improving and simplifying the techniques for cryopreserving the sperm of wild ungulates and thus facilitate their use under field conditions [1]. Iberian ibex is a mountain caprine endemic to the Iberian Peninsula that display marked vulnerability to environmental and sanitary factors. It has a short breeding season that extends from December to February, although spermatogenic activity remains through the year with the lowest sperm quality in spring [2]. The successful use of assisted reproduction technologies with this species is a useful model for *ex situ* conservation strategies designed to preserve other threatened wild ruminants [3]. Recent studies have reported live offspring of ibexes from artificial insemination with frozen and vitrified sperm [4, 5, 6].

Cryoinjury during conventional freeze-thawing is caused by factors such as thermal shock, ice formation, dehydration, increased salt concentration and osmotic shock [7, 8]. The cooling rate affects the extracellular ice formation; the cells and the dissolved salts are excluded from the ice and become concentrated in the unfrozen fraction remaining between the growing ice masses [9]. Thus, the osmotic strength of the unfrozen fraction increases, causing an efflux of water from the cells, resulting in cell shrinkage [10]. While conventional freezing requires the use of cryoprotectants to prevent damage caused by ice crystal formation, vitrification techniques allow glass transition and forms and stable structure without the presence of ice crystals through high concentrations of permeable cryoprotective agents [11] and/or very rapid freezing rates (kinetic vitrification) [12]. Sperm vitrification was first developed for use with humans as a means of simplifying and speeding up cryopreservation without the need for sophisticated equipment, making it cheaper than traditional protocols [12, 13]. High concentrations of cryoprotectant are harmful to sperm cells [5]; sperm vitrification, however, involves ultra-rapid cooling of small-volume samples without permeable cryoprotectant [14]. Rapid cooling rates should prevent formation of ice crystals inside the cells, and thus, the entire cell suspension should vitrify [15]. Some non-permeable additives with cryoprotective activity, such as human serum albumin and sucrose, have been successfully used in the kinetic vitrification of human sperm [16]. This modified method was subsequently used to successfully cryopreserve dog [17] and fish sperm [18], and more recently Iberian ibex (*Capra pyrenaica*) [6] and European mouflon (*Ovis musimon*) [19] sperm. Vitrification occurs—or not—depending on the cooling rate, the viscosity of the solution, and the volume to be preserved [20]. However, the term 'vitrification', should be used only when both the intracellular milieu and the extracellular environment of the sperm cells become vitrified [16]. The most common ultra-rapid cooling technique is based on dropping small volumes of sperm suspension directly into liquid nitrogen [6, 21, 22]. To the best of our knowledge, however, no information is available regarding the formation—or not—of ice in the extracellular milieu when using this procedure. Extracellular ice is a primary cause of sperm damage during the cryopreservation process [23].

The plasmalemma and acrosomal membranes are major sites of cryopreservation-induced sperm damage [24]; this is certainly true for wild ruminant sperm vitrified in pellets [19]. The size of the sperm head can also be affected. Although in most of the species studied, sperm cells are made smaller by conventional freezing-thawing [25, 26, 27], a recent study that included observations on several wild species reported that cryopreservation at very high cooling rates may lead to an increased sperm head size after freezing-thawing [28]. Motility has also been reported lower in frozen-thawed vitrified sperm than in conventionally cryopreserved sperm [19]. Reduced mitochondrial activity usually underlies reduced kinetic activity [29], which is consistent with the reduced mitochondrial functionality seen in vitrified human sperm after thawing [21]. Alterations to the mitochondrial membrane, and ultrastructural damage to the axoneme, can be caused by intracellular and extracellular ice produced during

the freezing process, with the severity of the damages perhaps differing depending on the cryopreservation method used [30, 31].

The aims of the present study were therefore: 1) to determine whether the extracellular milieu of Iberian ibex sperm samples is vitrified during a specific ultra-rapid cooling protocol previously used in this species; and 2) to compare the damage caused to the species' sperm when cryopreserved by ultra-rapid cooling and slow cooling. Data were compared with these obtained using slow freezing by cryopreservation methods previously tested in ibexes. We used the more successfully procedure [32], which varied in cryoprotectants and even number of sperm per sample (straw) in comparison with ultra-rapid procedure in pellets.

## Material and methods

### Animals and sperm collection

Animals were handled according to procedures approved by the INIA Ethics Committee (Órgano Regulador de los Comités de Ética de Experimentación Animal) that specifically approved the design of the current study (reference number ORCEEA 2014/027; reference regional government PROEX 271/14) and were performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. Iberian ibexes were housed in captivity at the INIA Department of Animal Reproduction. All had born at the INIA facilities. They were kept in a 250-$m^2$ enclosure with partial roof cover. All were fed Visan K-59 (Visan Ind. Zoot. S.A, Madrid, Spain) containing 15% crude protein, 15.7% crude fibre, 4% crude fat, 10.6% crude ash, 0.5% Na. This commercial feed was supplemented with barley grain, barley straw, and dry alfalfa. Water and vitamin/mineral blocks were available *ad libitum*. To alleviate stress during experimental procedures, animals were accustomed to handling in a small restraining stall (2-$m^2$) in which anesthesia was administered. The animals were anesthetized using 50 μg/kg intravenous detomidine (Domosedan®) (Pfizer Inc., Amboise Cedex, France), 0.5 mg/kg ketamine hydrochloride (Imalgene-1000®) (Rhône Mérieux, Lyon, France) and 0.5 mg/kg tiletamine-zolazepam (Zoletil-100®) (Virbac España S.A., Barcelona, Spain). Anesthesia was maintained with 1.5% isoflurane (Isobavet®) (Intervet/Schering Plough Animal Health, Madrid, Spain) in oxygen (flow rate 2.5 L/min) administered via an endotracheal tube. Pulse oximetry and capnography were used to monitor the condition of the animals. During all manipulations the eyes were covered with a mask to further reduce stress.

Fourteen sperm samples were collected by transrectal ultrasound-guided massage of the accessory glands (TUMASG) [33] from six anesthetized Iberian ibexes. Briefly, ultrasound examination of the bulbourethral glands, the seminal vesicles, and the ampulla of the vas deferens was performed using real-time transrectal ultrasonography employing a 7.5 MHz linear array probe (Prosound 2, Aloka CO., LTD, Tokyo, 181–8622 Japan). TUMASG was performed with the ultrasonographic probe placed on the ampulla of the vas deferens, using a back-and-forth motion to favour the expulsion of the spermatozoa. If the animal did not ejaculate, electrical stimuli (0.2 mA lasting 6–8 s) were provided with an electroejaculator, with intermittent breaks for TUMASG; a maximum of 1–3 electrical stimuli were usually required. The electroejaculator used was a Lane Pulsator IIIZ model (Lane Manufacturing Inc., Denver, Colorado, USA) consisting of a rectal probe 2.5 cm in diameter and 20.5 cm in length. The process was monitored by ultrasound scanning of the ampulla of the vas deferens, verifying that the emptying of the glands was complete. Stimulation was halted when the echogenicity of the ampulla was compatible with its being empty.

The diluents and all materials coming into contact with the semen were maintained at 37˚ C. The volume of the ejaculates was measured using a micropipette (Gilson, Villiers Le Bel,

France). The percentage of motile sperm and the quality of motility were initially evaluated via phase contrast microscope (Zeiss, Oberkochen, Germany). Only those ejaculates with a sperm motility value of >50%, and a score of >2 on a motility scale of 0 (lowest) to 5 (highest), were used in the subsequent experimental work. The samples were separated into two fractions to be diluted with one of two experimental extenders prepared in-house (using reagent-grade chemicals purchased from Panreac Química S.A. [Barcelona, Spain] or the Sigma Chemical Co. [St. Louis, Missouri, USA] depending on the freezing method used) until reaching a final concentration of 100 x $10^6$ sperm/mL. The extender used for the conventional slow cooling samples was TCG (3.8% Tris [wt/vol], 2.2% citric acid [wt/vol], 0.6% glucose [wt/vol] + 6% egg yolk [vol/vol], plus glycerol at 5% [vol/vol]; osmolality: 1150 mOsm/kg). The extender for the ultra-rapid cooling samples was the same, with the glycerol substituted by 100 mM sucrose (osmolality: 486 mOsm/kg). The osmolality measured in the absence of cryoprotectants of the TCG extenders was 345 mOsm/kg. All solutions were adjusted to pH 6.8, with NaOH at room temperature.

## Sperm cryopreservation

The sperm samples cryopreserved by ultra-rapid cooling were cooled for 30 min at 5˚C and then plunged drop-by-drop (about 50 μL/drop) directly into liquid nitrogen as reported by Pradiee et al. [5]. Sperm samples cryopreserved by conventional slow cooling were cooled at 5ºC for 1 h, and then maintained at this temperature for 2 h (total equilibration therefore 3 h). Aliquots were then loaded into 0.25 mL straws and frozen by placing them in nitrogen vapour 5 cm above the surface of a liquid nitrogen bath for 10 min [32]. Twelve months later the above cryopreserved sperm pellets were thawed by placing them on a DPP70 thermo-regulated conical hotplate (INIA, Madrid, Spain) set at 60–65˚C, and the straws thawed in a water bath at 37˚C for 30 s. Previous reports in our lab showed fast warming to be important in preventing damage to ibex sperm cryopreserved at high cooling rates [5].

## Conventional sperm analysis by fluorescence and the computer-aided sperm analysis (CASA) system

Sperm variables were assessed before and after cryopreservation. Total sperm concentration was determined in fresh semen using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). The total percentage of motile sperm and the straight line velocity (VSL) were determined using a computer-aided sperm analysis system (CASA) (SCA, Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capacity [33]. A minimum of three fields and 500 sperm were assessed.

Morphometric analyses were made on 10 samples (fresh and frozen-thawed for each cryopreservation method). Smears were stained with Hemacolor® (Merck KGaA, Darmstadt, Germany) and assessed using the ISASv1 system (Projectes I Serveis R+D, Valencia, Spain). The sperm head dimensions length (L), width (W), area (A), and perimeter (P), were determined by examining 100 sperm cell heads [34].

The status of the plasma membrane, acrosome membrane and mitochondrial membrane was assessed in 10 frozen-thawed samples cryopreserved by each method, using an association of propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381) and Mitotracker Green FM (MITO, Invitrogen M7514, USA) following the method of Forero-Gonzalez et al. [35] with slight modifications. An aliquot of 150 μL of semen diluted in TALP stock medium at 25 x $10^6$ sperm/mL was mixed with 2 μL of PI (2 mg/mL), 1 μL of MITO (0.5 mM) and 50 μL of FITC-PNA,100 μg/mL) and incubated in the dark at 38.5˚C for 8 min. Live sperm were classified into eight

categories according to their plasmalemma, acrosomal and mitochondrial staining: 1) sperm with an intact plasmalemma, an intact acrosome and an intact mitochondrial membrane (IP-IA-IM); 2) sperm with an intact plasmalemma, an intact acrosome and a damaged mitochondrial membrane (IP-IA-DM); 3) sperm with an intact plasmalemma, a damaged acrosome and an intact mitochondrial membrane (IP-DA-IM); 4) sperm with an intact plasmalemma, a damaged acrosome and a damaged mitochondrial membrane (IP-DA-DM); 5) sperm with a damaged plasmalemma, an intact acrosome and an intact mitochondrial membrane (DP-IA-IM); 6) sperm with a damaged plasmalemma, an intact acrosome and a damaged mitochondrial membrane (DP-IA-DM); 7) sperm with a damaged plasmalemma, a damaged acrosome and intact mitochondrial membrane DP-DA-IM); 8) sperm with a damaged plasmalemma, a damaged acrosome and a damaged mitochondrial membrane (DP-DA-DM). A total of 200 sperm cells per sample were analyzed.

## *Cryo-scanning electron* microscopy *(Cryo-SEM)*

The Cryo-SEM was made in the Universitat Politècnica De València. The extracellular milieu of samples cryopreserved by the slow cooling (three samples [in straws]) and ultra-rapid cooling (three samples [in pellets]) methods was studied by Cryo-SEM. Cryopreserved samples of each type, containing about 25 million of sperm per straw and 5 million of sperm per pellet, maintained at all times in liquid nitrogen, were manually fractured; the straw samples were broken into sections 0.3–0.5 cm long, while the pellets were broken in half. All samples were then mounted on a mechanical cryo-transfer holder, maintaining them at -196ºC, and fixed with TBS Tissue Freezing Medium for frozen tissue specimens (Triangle Biomedical Sciences, Durham, NC, USA), which solidified in contact with the liquid nitrogen. The grip holder was transferred into a chamber containing snow nitrogen, and then moved into an Oxford CT1500 cryostage chamber (Oxford, UK) attached to a JEOL JSM 5410 Scanning Microscope (Japan). The temperature of sample is raised by heating the holder to -90ºC for 5–10 min in order to sublimate free water in the solid state lakes, followed by a temperature decrease to -130ºC to stabilize the sample. The coated sample was then transferred to the microscope chamber where it was analyzed at a temperature range of -125 to -135ºC. The glass transition temperature is raised over -90º C in hypertonic solutions (e.g. sucrose-egg yolk based extender for the ultra-rapid cooling), and thus the frozen state was not affected in the sublimation step [9, 36]. The frozen preparation was then gold-sputtered. Digital images were obtained at 15 kV using an Oxford CT500 camera running INCA Oxford software (Oxford, UK), at magnifications of 750–3500. As a control, straws containing a conventional extender for vitrifying embryos (a final solution containing 20% ethylene glycol and 20% DMSO as cryoprotectants) [37] were analyzed to compare its frozen state with the test samples. Straw sections of 1 cm long were cut, placed on the cryo-transfer holder and analyzed as above. ImageJ v.1.8.0 software (National Institutes of Health (NIH), Maryland, USA) was used to determine the size of the crystals produced by each cooling method. The crystal perimeter and four crystal shape descriptors were also recorded: *circularity* ($4\pi \times [\text{Area}]/[\text{Perimeter}]^2$) (a value of 1.0 indicates a perfect circle; values approaching 0.0 indicate an increasingly elongated shape [values may not be valid for very small crystals]; *aspect ratio* of the crystals' fitted ellipse, i.e., [Major Axis]/[Minor Axis]; *roundness*, i.e., the inverse of the aspect ratio; and *solidity* ([Area][Convex area]).

## Scanning electron microscopy (SEM)

The SEM was made in the ICTS-Centro Nacional de Microscopía Electrónica from the Universidad Complutense of Madrid. Cryopreserved straws and pellets (four samples for each

method) were thawed as described in sperm cryopreservation section, transferred to 750 μl of a fixative solution (4% paraformaldehyde and 2.5% glutaraldehyde), maintained for 4 h at 5˚C, and filtered using fluoropore filters (13 mm diameter and pore size 0.2 μm) (Merck Millipore Ldt. Cork, Ireland). The sperms remaining on the filters were washed (twice) directly over using distilled $H_2O$ and then, filters were introduced in a test tube containing distilled water for 10 min. After dehydration through an ascending series of alcohol solutions, the material was critical point dried using a Leica EM CPD 300 critical point dryer (Leica, Wetzlar, Germany). Then, they were gold-sputtered and examined using a Jeol JSM 6400 electron microscope (Tokyo, Japan). Images were captured using INCA Oxford software (Oxford, UK) and the sperms examined for structural damage to the sperm head, acrosome membrane and midpiece.

### Transmission electron microscopy (TEM)

The TEM was made in the ICTS-Centro Nacional de Microscopía Electrónica from the Universidad Complutense of Madrid. Four frozen-thawed samples of sperm cryopreserved by the two methods were diluted 1:9 (vol/vol) with a wash medium composed of 3.8% Tris (wt/vol), 2.2% citric acid (wt/vol), 0.6% glucose (wt/vol), and centrifuged at 1.2 *g* for 15 min. The supernatant was then removed and the pellet resuspended and fixed in 2 ml of Karnovsky solution at 5˚C for 4 h. This was followed by washing with Milloning buffer 1.2 g for 3 x 15 min, postfixing in 1% $OsO_4$ for 1 h, and washing in miliQ water (3 x 10 min). The samples were then dehydrated in an ascending series of acetone plus SPURR resin (1:3 for 1 h, 1:1 for 1 h and 3:1) overnight at 65–70˚C. Ultrathin sections were cut using a Reicher Ultracut S ultramicrotome (Leica, Wetzlar, Germany) and examined on copper grids with 200 hexagonal meshes using a JEM 1400 electron microscope (Tokyo, Japan) equipped with a Digital Micrograph image analysis system (Gatan, California, USA). Images of sperm cells were assessed qualitatively for changes in plasmalemma, mitochondrial and midpiece ultrastructure.

### Statistical analysis

Data are expressed as means ± SE. The normality of data distribution was examined using the Shapiro–Wilks test; non-normal values were arcsine-transformed before analysis. The paired t-test was used to compare the effect of cryopreservation method on the examined sperm variables within and between cryopreservation methods. A total of 14 sperm samples were analysed.

GLM nested ANOVA was used to compare cryopreservation methods in terms of the shape and size of the ice crystals produced, following the statistical model $X_{ij} = m + T_i + A_{j(i)} + e_{ijk}$, where $X_{ij}$ = accuracy of the assay, m = overall means of crystals variables, $T_i$ = cryopreservation method (i = 1–2), $A_{j(i)}$ = effect of the number of samples (j = 1–3 for $T_1$ and j = 1–3 for $T_2$), and $e_{ijk}$ = residual (k = 1–100).

Where applicable, significance was set at $p<0.05$. All analyses were performed using STATISTICA software for Windows v.12.0 (StatSoft, Inc., Tulsa, OK, USA).

## Results

### Sperm evaluations by CASA and fluorescence microscopy

The fresh sperm samples had 78.43 ± 4.17% viable sperms, 94.71 ± 0.97% sperms with an integral acrosome, and 65.04 ± 5.16% motile sperms. The straight line velocity (VSL) of these sperms was 40.20 ± 4.10 μm/s. After freezing-thawing, the percentage of motile sperms and the VSL values were higher for the samples cryopreserved by slow cooling (22.95 ± 3.22% *vs*

**Table 1. Morphometric values (mean ± SE) for fresh and thawed Iberian ibex sperm subjected to either slow conventional freezing or ultra-rapid cryopreservation.**

| | Fresh | Post conventional freezing | Post ultra-rapid freezing |
|---|---|---|---|
| Length (μm) | 8.42 ± 0.06[a] | 8.15 ± 0.08[b] | 8.17 ± 0.07[b] |
| Width (μm) | 4.27 ± 0.03[a] | 4.30 ± 0.04[a] | 4.25 ± 0.02[a] |
| Area ($\mu m^2$) | 29.71 ± 0.29[a] | 28.60 ± 0.53[a] | 28.59 ± 0.17[b] |
| Perimeter (μm) | 22.48 ± 0.13[a] | 21.97 ± 0.19[b] | 21.98 ± 0.12[b] |

Different lower case letters (a, b) indicate significant differences (P < 0.05) between fresh samples and post-thawing after each cryopreservation method.

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4.42 ± 0.86% for those cryopreserved by ultra-rapid cooling, and 38.06 ± 3.45 μm/s *vs* 25.11 ± 3.96 μm/s for those cryopreserved by ultra-rapid cooling; P<0.001 and P<0.02 respectively). For both cryopreservation techniques, the sperm head dimensions were smaller after freezing-thawing compared to fresh sperm (P<0.05 for both methods) (Table 1).

The percentage of sperm with intact plasmalemma, intact acrosome and with intact mitochondrial membrane (IP-IA-IM category sperm) was greater after slow cooling than after ultra-rapid cooling (Table 2; P<0.01), while the percentage of sperm with damaged plasmalemma, damaged acrosome and damaged mitochondrial membrane (DP-DA-DM category sperm) was greater after ultra-rapid cooling than after slow cooling (Table 2; P<0.01). The total percentage of sperm with intact mitochondria, the total percentage with an intact plasma membrane, and the total percentage with an intact acrosome, was greater after slow cooling than after ultra-rapid cooling (P<0.05) (Table 2; Fig 1).

**Table 2. Percentage of sperm with intact/damaged plasmalemma, acrosome, and mitochondrial membrane of Iberian ibex sperm subjected to either slow conventional freezing or ultra-rapid cryopreservation.** Values are mean ± SE.

| | Post slow freezing | Post ultra-rapid freezing |
|---|---|---|
| IP-IA-IM (%) | 14.01 ± 2.83[a] | 3.20 ± 0.57[b] |
| IP-IA-DM (%) | 11.08 ± 2.04[a] | 9.30 ± 1.92[a] |
| IP-DA-IM (%) | 0.25 ± 0.13[a] | 0.10 ± 0,10[a] |
| IP-DA-DM (%) | 0.3 ± 0.21[a] | 0.20 ± 0.20[a] |
| DP-IA-IM (%) | 1.1 ± 0.69[a] | 0.20 ± 0.13[a] |
| DP-IA-DM (%) | 15.26 ± 2.20[a] | 14.3 ± 2.26[a] |
| DP-DA-IM (%) | 1.16 ± 0.67[a] | 0.50 ± 0.16[a] |
| DP-DA-DM (%) | 56.82 ± 4.15[b] | 72.20 ± 1.80[a] |
| Total IM (%) | 16.52 ± 3.75[a] | 4 ± 0.65[b] |
| Total IP (%) | 25.64 ± 3.71[a] | 12.8 ± 2.50[b] |
| Total IA (%) | 41.45 ± 3.73[a] | 27 ± 1.84[b] |

Different lower case letters (a, b) indicate significant differences between cryopreservation methods. IP-IA-IM: sperm with intact plasmalemma, intact acrosome and with intact mitochondrial membrane; IP-IA-DM: sperm with intact plasmalemma, intact acrosome and damaged mitochondrial membrane; IP-DA-IM: sperm with intact plasmalemma, damage acrosome and intact mitochondrial membrane; IP-DA-DM: sperm with intact plasmalemma, damaged acrosome and damaged mitochondrial membrane. DP-IA-IM: sperm with damaged plasmalemma, intact acrosome and with intact mitochondrial membrane; DP-IA-DM: sperm with damaged plasmalemma, intact acrosome and damaged mitochondrial membrane; DP-DA-IM: sperm with damaged plasmalemma, damaged acrosome and intact mitochondrial membrane; DP-DA-DM: sperm with damaged plasmalemma, damaged acrosome and damaged mitochondrial membrane. IM: total sperm with intact mitochondrial membrane; IP: total sperm with plasmalemma integrity; IA: total sperm with intact acrosome.

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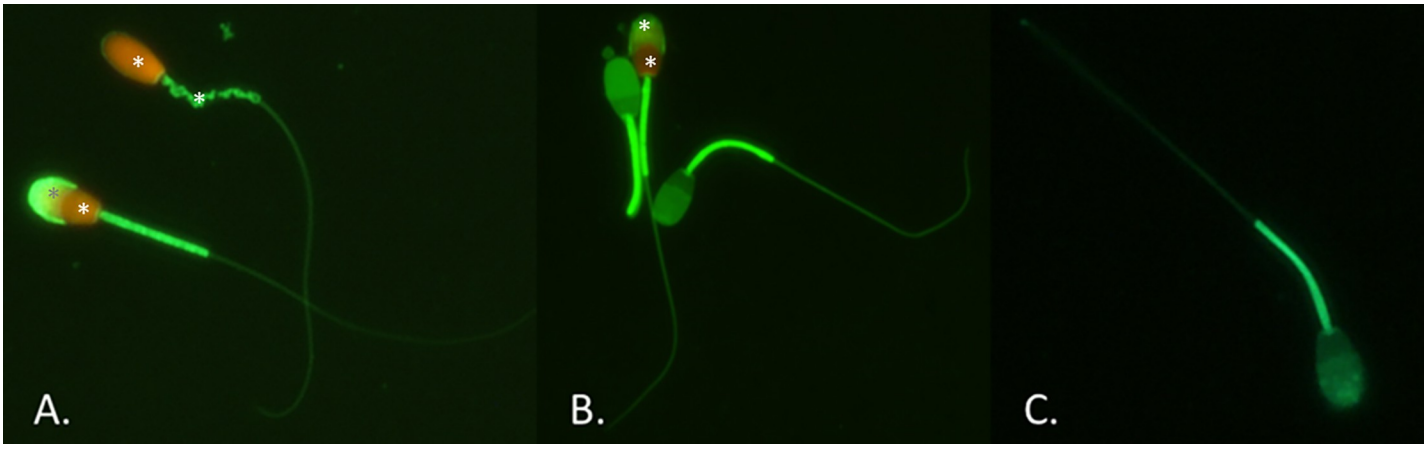


**Fig 1. Sperm stained with an association of propidium iodide, fluorescein isothiocyanate-conjugated peanut agglutinin and Mitotracker Green FM (x400).** A. Dead sperm cell with a damaged plasmalemma, an intact acrosome and a damaged mitochondrial membrane (DP-IA-DM, on top), and a dead sperm cell with a damaged plasmalemma, a damaged acrosome and an intact mitochondrial membrane (DP-DA-IM, on bottom). B. Two viable sperm cells with an intact plasmalemma, an intact acrosome and an intact mitochondrial membrane (IP-IA-IM), and one dead sperm cell (in the middle) with a damaged plasmalemma, a damaged acrosome and an intact mitochondrial membrane (DP-DA-IM). C. Viable sperm cell with an intact plasmalemma, an intact acrosome and an intact mitochondrial membrane (IP-IA-IM). Asterisks indicate the sperm damages.

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## Cryo-SEM

The Cryo-SEM appearance of the frozen, slow-cooled and ultra-rapid cooled extenders differed from that of the frozen control extender used to vitrify embryos. The latter appeared totally homogeneous, while, both the frozen test extenders showed 'lakes' (small areas occupied by pure ice) and veins, i.e., areas with high solute concentrations (Fig 2). Thus, Cryo-SEM revealed ultra-rapid cooling not to induce a stable, glass-like extracellular state. Nevertheless, after ultra-rapid cooling, the crystals were smaller ($P<0.001$ for both perimeter and area aspect ratio) and more stretchmarked than those observed after slow cooling. The circularity and roundness values of the ultra-rapid cooling-associated crystals were higher ($P<0.001$ for each variable). However, no differences were seen in the solidity of the crystals produced by the two techniques (Table 3). Freeze sperm cells entrapped in the freeze matrix were occasionally observed without evident intracellular ice formation (Fig 2D).

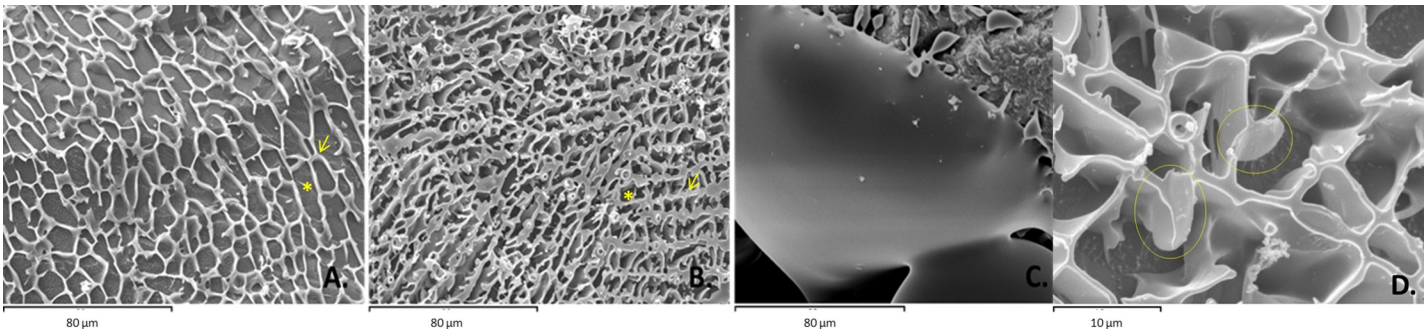


**Fig 2. Cryo-SEM images of the distribution of ice crystals within the extender matrix (mag. x750). A.** Cryopreservation state after slow cooling in straws with 5% glycerol as a cryoprotectant. **B.** Cryopreservation state after ultra-rapid cooling as pellets (50 μl) with 100 mM sucrose as a cryoprotectant. **C.** Cryopreservation state of a conventional medium used for embryo vitrification (control). Asterisks showed the lakes and arrows showed the veins. **D.** Two sperm heads (circles) entrapped in the freeze matrix, without evident intracellular ice formation.

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**Table 3. Size and shape descriptors (values are mean ± SE) of crystals assessed by image analysis for both cryopreservation methods.**

| | Size and shape descriptors of crystals | |
|---|---|---|
| | **Slow freezing** | **Ultra-rapid freezing** |
| **Area (μm²)** | 45.93 ± 3.96[a] | 8.98 ± 0.00[b] |
| **Perimeter (μm)** | 29.57 ± 2.05[a] | 12.95 ± 0.00[b] |
| **Aspect ratio** | 0.66 ± 0.01[a] | 0.65 ± 0.00[b] |
| **Circularity** | 2.18 ± 0.05[b] | 2.20 ± 0.00[a] |
| **Round** | 0.56 ± 0.00[a] | 0.52 ± 0.00[b] |
| **Solidity** | 0.82 ± 0.00[a] | 0.79 ± 0.00[a] |

Different lower case letters (a, b) indicate significant differences between crystals found during slow and ultra-rapid freezing states. Shape descriptors (aspect ratio, circularity, round, solidity) are not expressed in units.

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## SEM and TEM evaluations

With SEM, no significant differences were seen between the cooling methods in terms of damage to the sperm head, acrosome membrane, or mitochondrial structure. In the slow cooled samples, some midpieces appeared to be swollen, causing some mitochondrial damage (Fig 3A), and some regions of the acrosomal membrane showed perforations (Fig 3B). In the ultra-rapid cooled samples some sperms showed a wrinkled (Fig 3C) or swollen (Fig 3D) acrosome ridge membrane, rolled tails, and broken midpieces (Fig 3C).

Some sperms subjected to slow cooling showed an intact acrosome membrane but a shrunken (Fig 4A) and blebbing plasma membrane (Fig 4B). Some sperms subjected to ultra-rapid cooling showed a swollen or disintegrated acrosome and plasma membranes (Fig 4C). The acrosome membrane also appeared reacted in some cases.

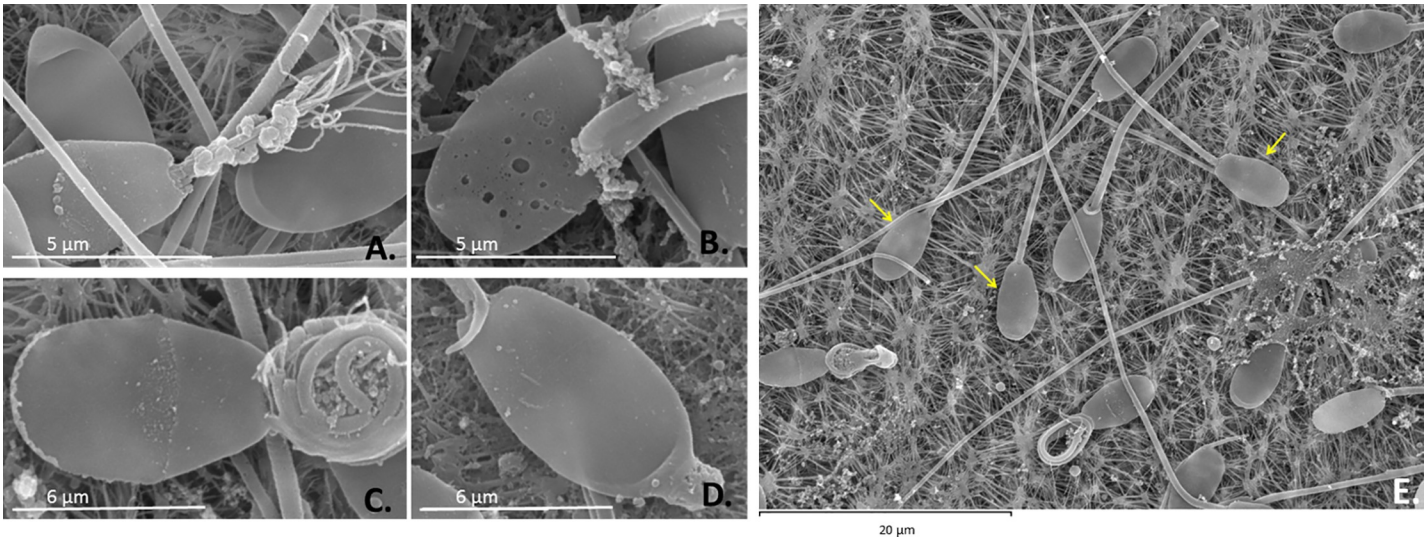


**Fig 3. SEM images showing structural damage to thawed ibex sperm after both cryopreservation processes.** A and B. Sperm cells after slow cooling with 5% glycerol cryoprotectant. Notice the swollen midpiece damaging the mitochondria (A) and the disruption of the perforated acrosomal membrane (B). C and D. Sperm cells after ultra-rapid cooling with 100 mM sucrose cryoprotectant. Notice the acrosome ridge membrane is wrinkled, the tail rolled, and the midpiece broken. In D, the apical ridge of the acrosome is somewhat swollen (mag. x8500). E. Sperm cells after ultra-rapid cooling. Notice some sperm cells which successfully achieved the cryopreservation process (arrows) (mag. x2500).

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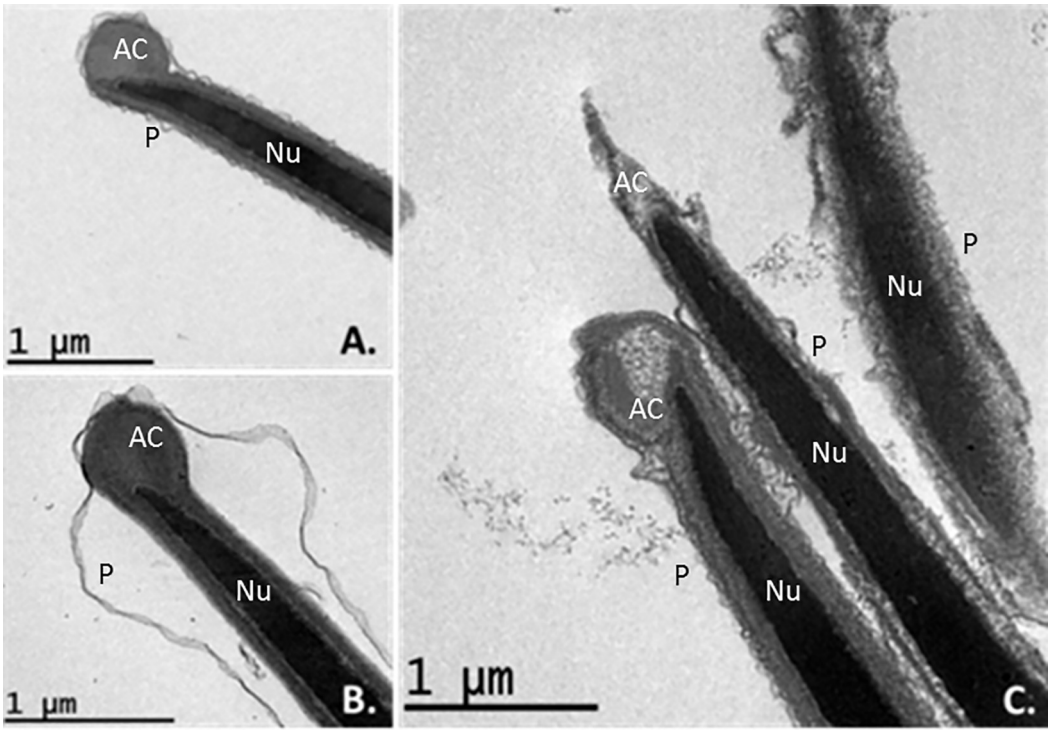


**Fig 4. TEM images of osmotically induced pathological changes in transverse and longitudinal sections of thawed ibex sperm heads.** Damage to the acrosome membrane is particularly clear irrespective of the cooling rate employed. A and B. Sperm heads after slow-freezing showing an intact acrosome membrane but a shrunken plasma membrane (A) and membrane blebbing (B) (mag. x3000 and x4000). **C.** Sperm head membranes after ultra-rapid freezing. Notice the plasma and acrosome head membranes are reacted, swollen and disintegrated (mag. x3000). Nu = nucleus, AC = acrosome membrane, P = plasma membrane.

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Under TEM, slow cooling was associated with many sperms showing a swollen plasmalemma in the acrosomal and post-acrosomal head regions in longitudinal section. The same was observed in transverse sections of the tails and midpieces, in which the mitochondria showed notable vacuolization (Figs 5A and 6A and 6C). Ultra-rapid cooling was associated with many sperm heads showing no plasmalemma (it had disintegrated) in longitudinal section (Figs 5B and 6B); some heads and acrosome membranes also appeared broken (Figs 5B and 6B and 6D). Although many mitochondria showed vacuolization, this was less notable than with slow cooling (Figs 5 and 6). In addition, some sperm subjected to ultra-rapid cooling showed membrane detachment along the tail (Fig 6D), and an unstructured axoneme (Figs 6D and 7). Overall, ultra-rapid cooling seemed to be associated with the midpiece being smaller in volume; this was not so common for the slow cooled samples (Figs 5 and 6).

Unstructured axonemes of the tail were observed with both cryopreservation methods. Displacements of microtubules were more usual in sperm samples conventionally frozen (S1 Fig).

## Discussion

This is the first report to assess the ultrastructural damage to ibex sperm cells after cryopreservation. It is suggested by Cryo-SEM method that ultra-rapid cooling failed to induce a stable, glass-like state in the extracellular milieu, but the ice crystal morphology was different to that seen in samples cryopreserved by conventional slow cooling. The sperm cryopreserved by the ultra-rapid cooling method had lower motility values and suffered greater damage to the

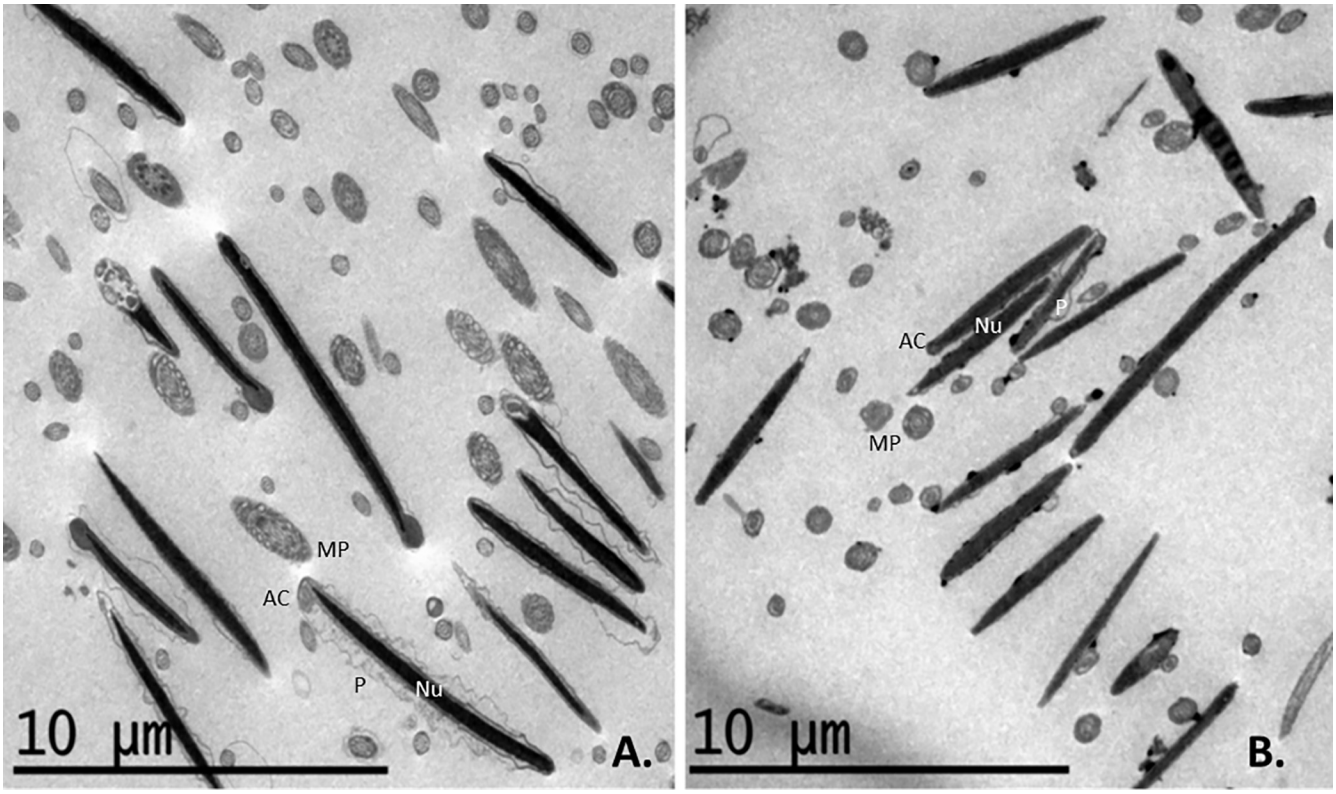


**Fig 5. TEM images of transverse and longitudinal sections of thawed ibex sperm following slow and ultra-rapid cooling (mag. in x500).** A. Sperm internal structures after slow cooling. Most longitudinal sections of the sperm heads show a swollen plasmalemma in the acrosomal and post-acrosomal region. In transverse sections of the midpiece, the mitochondria show a great deal of vacuolization. B. Sperm internal structures after ultra-rapid cooling. Most of the longitudinal sections of the sperm heads show no plasmalemma since it has disintegrated; some heads and acrosome membranes appear broken. Many mitochondria show vacuolization. The volume of the midpiece is smaller after ultra-rapid cooling (B) than slow cooling (A). Nu = nucleus, AC = acrosome membrane, P = plasma membrane, MP = midpiece.

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plasmalemma, acrosome membrane and mitochondrial membrane. Maybe the rapid cooling in the phase prior to ice nucleation could lead to cold shock damage, characterised by a leakage of solutes across to the extracellular milieu, associated with lipid phase transitions in cell membranes [38].

An issue to consider is the possibility that vitrified samples could crystallize during the process of heating to -90ºC, which is made to sublimate free water in the solid state lakes during cryo-SEM procedure. Although estimative methods of glass transition on simple aqueous solution containing carbohydrates could support this circumstance [39], the extender used in the present study also contained Tris, citric acid, and even proteins from the egg yolk. In addition glassy state has a near null sublimation rate, so vitrified solutions do not present enhanced contrast after the etching process [40, 41]. In the same way many reports [42, 43, 44] showed that cryo-SEM allows observations of microstructural features with no alteration due to preparation procedures. All this studies support the use of cryo-SEM to determine the glassy state in cryopreservation process. However, our findings results are only applied to this specific cryopreservation method used, with its own characteristics (e.g. pellet volume, diluent composition, etc.) that may differ from others.

The present results did not reveal evidence of any intracellular ice in the head sperm entrapped in the freeze matrix with both ultra-rapid and slow cooling rates. This agree with other reports using different cooling rates in which examined sperm do not contain

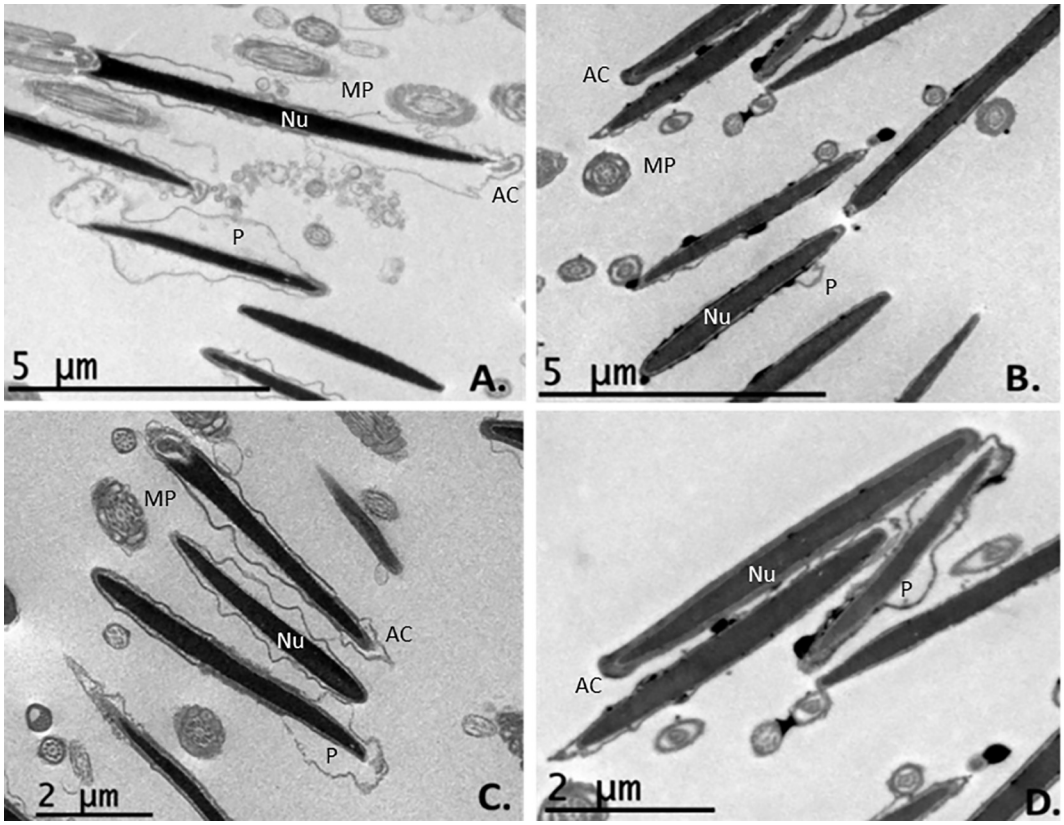


**Fig 6. TEM images of transverse and longitudinal sections of thawed ibex sperm following slow and ultra-rapid cooling.** A. Sperm structures after slow cooling; transverse sections of sperm heads with some damage to the head membranes and vacuolization of the mitochondria (mag. x1000). B. Sperm structures after ultra-rapid cooling; note the damage to the plasmalemma (sometimes entirely lost) and the vacuolization of the mitochondria (mag. x1000). C. Sperm head and midpiece sections with different plasmalemma damage and mitochondrial vacuolization states after slow cooling (mag. x1200); D. Sperm head and midpiece sections with different degrees of damage to the plasmalemma, membrane detachment along the tail, mitochondrial vacuolization and axoneme destructuring following ultra-rapid cooling (mag. x1500 magnification). The midpiece volume seems smaller after ultra-rapid cooling (B, D) than after slow cooling (A, C). Nu = nucleus, AC = acrosome membrane, P = plasma membrane, MP = midpiece.

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intracellular ice i.e. vitrification of the intracellular compartment always occur [9, 45, 46]. However, small homogeneous ice crystals may not be observed by this SEM technique, and thus only the absence of large ice crystals can be shown. The present study shows that there are not clear evidences for intracellular ice formation within the sperm head, unlike larger cells (e.g. oocytes and embryos). Not all studies support the idea that intracellular ice forms even when using slow cooling rates (which theoretically should be more likely) [45, 47]. Thus, intracellular vitrification (in which no crystals form) may be quite easy to achieve in sperm cells [12] even by conventional slow cooling [9], a likely consequence of their small size and high soluble macromolecule content. Certainly, it is well known that vitrification depends on the interaction between the cooling rate, the viscosity and volume of the solution [20].

It has also long been assumed that few crystallization nuclei form in the extracellular milieu during the ultra-rapid cooling of small volumes, and that any that do are too small to damage cells or cause any substantial shrinkage [12]. However, the present findings do not support this idea; the ultra-rapid freezing rate did not prevent the formation of ice crystals in the extracellular milieu. In the samples thus treated, severe membrane damages was observed, and the changes recorded in the sperm head dimensions suggest the cells suffered shrinkage and then

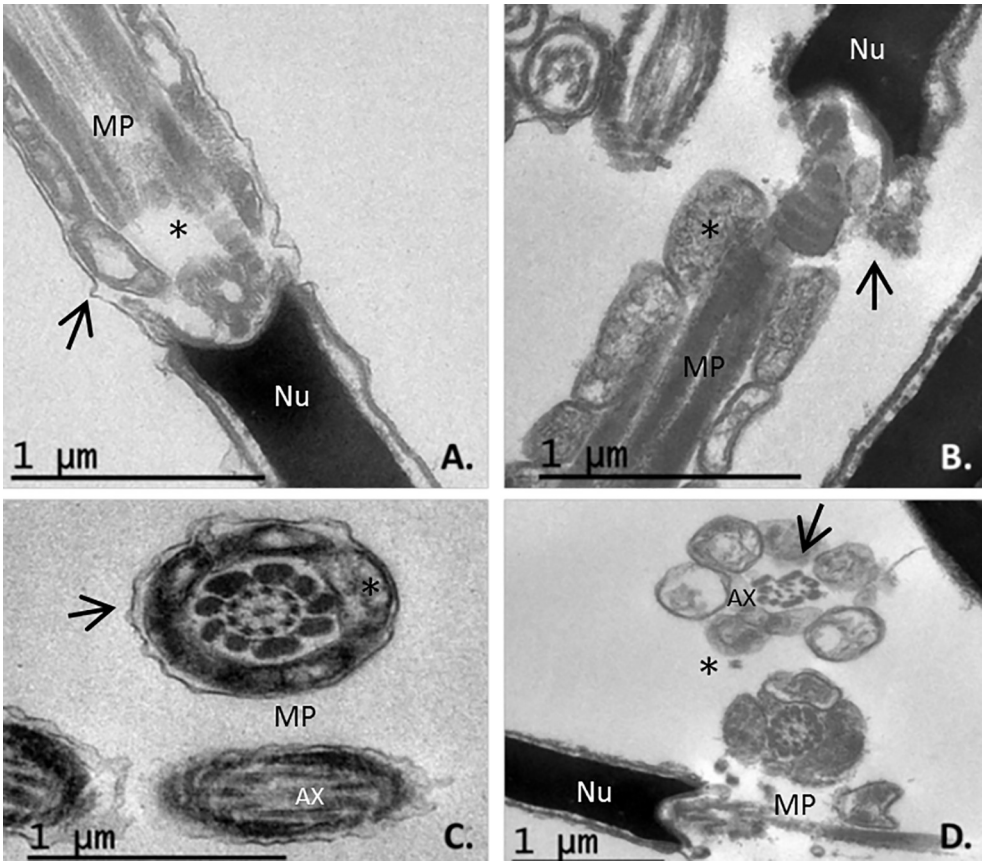


**Fig 7. TEM images of osmotically induced pathological changes in transverse and longitudinal sections of the midpiece in thawed ibex sperm after slow and ultra-rapid cooling.** A and C. Mitochondria showing vacuolization (asterisks) and detachment of the midpiece plasma membrane (arrows) were seen after slow cooling (mag. x5000). B and D. Sperm cell showing an unstructured midpiece and axoneme (arrows), plus mitochondria showing vacuolization (asterisks), after ultra-rapid cooling (mag. x5000 and x3000). Nu = nucleus, AC = acrosome membrane, P = plasma membrane, MP = midpiece, AX = axoneme.

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swelling over the freezing-thawing process. However, these findings should be understood with caution since there is scant information on this technique, and the volume used in the present study (50 μL) was greater than that used in other studies to develop kinetic vitrification (20 μL) [48]. Differences in the cooling rate may also help explain the differences seen in the shape descriptors and size of the crystals formed during ultra-rapid and slow cooling [49]. Extender composition might also affect the crystallization process. Sucrose is thought to slow down ice nucleation [49, 50]; the idea that it may influence the shape of any ice crystals formed cannot, therefore, be ruled out. After ultra-rapid cooling, the crystals were smaller and more stretchmarked than those observed after slow cooling. This pattern of ice formation in ultra-rapid method might favour a direct contact of ice with cells, producing a greater damage.

Differences in the characteristics of the ice crystals in the extracellular milieu produced by the tested methods might lead to differences in the abundance of pockets of high solute concentration, subjecting the cells to different levels of osmotic stress. It is well known that cryopreservation reduces sperm in motility and viability, and that it modifies cell morphometric variables. Such damage might be related to the osmotic stresses resulting from the water–solute interactions that arise because of crystallization [51]. As dissolved salts are excluded from the forming ice, they become concentrated between ice crystals forming pockets of high solute

concentration. These would draw out of cells, leading to their shrinkage, and eventually bursting of the membrane [52]. Although extracellular ice seems a primary cause of sperm damage during the cryopreservation process [23], some intracellular ice formation cannot be ruled out, in cell compartments other than the head, as sperm midpiece and tails [53, 54]. Indeed, our findings showing the displacements of microtubules of tail (S1 Fig) might be produced by ice crystals. Reduction in sperm motility might be related to damage caused to the mitochondrial membranes [55], which was more severe in the present ultra-rapidly cooled sperms (as shown by MITO-fluorescence analysis). Some sperm morph-structural variables, such as the status of the plasma membrane, acrosome membrane and mitochondrial membrane are related directly with the functionality of sperm cells. The last correlates with the sperm motility, but the others two are not necessary for this parameter, being related with others fertility functions. Sperm cells with damaged acrosome and/or plasmalema are non-functional and thus are not considered to be successfully preserved, even if they have intact mitochondria and are able to move. The term of "motile sperm" includes total progressive and non-progressive sperm. Certainly, the percentage of sperm with intact plasmalema, acrosome and mitochondrial membranes (IP-IA-IM, 14%) were lower than the percentage of motile sperm (23%) in samples by slow cooling. This fact reveals that sperm with the plasmalema damaged may show kinetic activity if the mitochondria still remains unaltered. Even though the percentage of intact mitochondrial membranes was less than percentage of motile sperm for slow conventional freezing, the results are in agreement with other study about bovine sperm cryopreservation [35]. The damage caused to the mitochondrial membrane during slow cooling and later thawing, which affected about 83% of the sperm cells, was probably caused by swelling (Fig 3A) and vacuolization (Figs 5A and 6A and 6C). These quantitative changes were accompanied by differences in the ultrastructural changes suffered by the mitochondria (as shown by SEM and TEM). The ultra-rapid cooling technique affected 96% of sperm mitochondria (as shown by fluorescence), perhaps caused by mitochondrial vacuolization (this value fell to 83% with slow cooling). TEM also revealed the midpiece to have a smaller volume after ultra-rapid cooling than after slow cooling, suggesting an inability to return to initial volumes after osmotic shock, perhaps due to irreversible damage to the plasma membrane [10]. This would explain the strong reduction in motility when ultra-rapid cooling is employed [19].

The SEM results show that, with both freezing methods, some sperm suffer a fractured plasmalemma and a disrupted acrosome membrane. Similar damage has been encountered in sperm samples from other species after freezing-thawing, e.g. in goats [56], cattle [57] and the blue fox [58]. Krogenaes et al. [57] report that, in TEM images of frozen-thawed bull sperm, the plasmalemma appeared swollen and detached from the sperm head. Such damage, along with vacuolization of the mitochondria, might be associated with osmotic shock [10] suffered during freezing-thawing. Ultrastructural changes in the acrosomes and mitochondria were described as similar to those seen in bovine and ovine sperm frozen by conventional techniques [59, 60]. González-Fernández et al. [10] also suggest that all these kinds of ultrastructural alteration in equine sperm are likely produced by osmotic changes. In addition, they report that under hypotonic conditions (75 mOsm), TEM images showed the sperm cell plasmalemma to be swollen and detached from the sperm head, and mitochondrial to be volume increased; the mitochondrial volume decreased under hypertonic conditions. Similar osmotic changes might therefore be expected during freezing-thawing.

In conclusion, ultra-rapid cooling of ibex sperm by spheres is a promising and useful alternative to conventional freezing methods, mainly when sperm cryopreservation is performed in wild species under field conditions with lack of sophisticated equipment. This technique did not prevent the production of extracellular ice; the extracellular milieu did not achieve a truly vitrified state. Although ultra-rapid cooling simplifies the process of cryopreservation and it

has been successfully used in other wild species [28], it causes severe damage to sperm cell membranes, and thus effort should be focused to investigate new additives-cryoprotectants and to optimize the volume of pellets for avoiding the extracellular ice formation.

## Supporting information

**S1 Fig. TEM images showing structural damage to ibex sperm axonemes.** Notice the displacements of microtubules (arrows) and vacuolization of mitochondria (asterisks).
(TIF)

## Author Contributions

**Conceptualization:** Milagros Cristina Esteso, Julián Santiago-Moreno.

**Formal analysis:** Paula Bóveda, Cristina Castaño, Julián Santiago-Moreno.

**Funding acquisition:** Milagros Cristina Esteso, Antonio López-Sebastián, Julián Santiago-Moreno.

**Investigation:** Adolfo Toledano-Díaz, Milagros Cristina Esteso, Antonio López-Sebastián, Dimitrios Rizos, Alejandro Bielli, Rodolfo Ungerfeld, Julián Santiago-Moreno.

**Methodology:** Paula Bóveda, Adolfo Toledano-Díaz, Cristina Castaño.

**Supervision:** Julián Santiago-Moreno.

**Visualization:** Antonio López-Sebastián.

**Writing – original draft:** Paula Bóveda, Milagros Cristina Esteso, Dimitrios Rizos, Alejandro Bielli, Rodolfo Ungerfeld, Julián Santiago-Moreno.

**Writing – review & editing:** Julián Santiago-Moreno.

## References

1. Pradiee J, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, Santiago-Moreno J. Cryopreservation of epididymal sperm from ibexes (*Capra pyrenaica*) using short equilibration time with glycerol. Theriogenology. 2014; 82(3):525–528. https://doi.org/10.1016/j.theriogenology.2014.05.012 PMID: 24938799
2. Coloma MA, Toledano-Díaz A, Castaño C, Velázquez R, Gómez-Brunet A, López-Sebastián A, et al. Seasonal variation in reproductive physiological status in the Iberian ibex (*Capra pyrenaica*) and its relationship with sperm freezability. Theriogenology. 2011; 76:1695–1705. https://doi.org/10.1016/j.theriogenology.2011.07.001 PMID: 21855981
3. Santiago-Moreno J, Coloma MA, Toledano-Díaz A, Castaño C, Gómez-Brunet A and López-Sebastián A. Assisted reproduction in Mediterranean wild ruminants: lessons from the Spanish ibex (*Capra pyrenaica*). Soc. Reprod.Fertil. Suppl. 2010; 67: 431–441. PMID: 21755689
4. Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Gómez-Brunet A, López-Sebastián A. Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived by artificial insemination with epididymal spermatozoa retrieved after death. Theriogenology. 2006; 66:283–91. https://doi.org/10.1016/j.theriogenology.2005.11.012 PMID: 16376980
5. Pradiee J, Esteso MC, López-Sebastián A, Toledano-Díaz A, Castaño C, Carrizosa JA, et al. Successful ultra-rapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa. Theriogenology. 2015; 84:1513–1522. https://doi.org/10.1016/j.theriogenology.2015.07.036 PMID: 26316218
6. Pradiee J, Sánchez-Calabuig MJ, Castaño C, O'Brien E, Esteso MC, Beltran-Breña P, et al. Fertilizing capacity of vitrified epididymal sperm from Iberian ibex (*Capra pyrenaica*) Theriogenology. 2018; 108:314–320. https://doi.org/10.1016/j.theriogenology.2017.11.021 PMID: 29288975
7. Mazur P. Freezing of living cells: mechanisms and implications. Am. J. Physiol. Physiol. 1984; 247:125–142.

8. Woelders H, Matthijs A, Engel B. Effects of Trehalose and Sucrose, Osmolality of the Freezing Medium, and Cooling Rate on Viability and Intactness of Bull Sperm after Freezing and Thawing. Cryobiology. 1997; 35:93–105. https://doi.org/10.1006/cryo.1997.2028 PMID: 9299101
9. Morris GJ, Acton E, Murray B, Fonseca F. Freezing injury: The special case of the sperm cell. Cryobiology. 2012; 64:71–80. https://doi.org/10.1016/j.cryobiol.2011.12.002 PMID: 22197768
10. González-Fernández L, Morrell JM, Peña FJ and Macías-García B. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology. 2012; 78:415–422. https://doi.org/10.1016/j.theriogenology.2012.02.021 PMID: 22578615
11. Pegg DE. Principles of cryopreservation. Methods Mol Biol. 2015; 1257:3–19. https://doi.org/10.1007/978-1-4939-2193-5_1 PMID: 25428001
12. Isachenko E, Isachenko V, Katkov I, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reprod Biomed Online. 2003; 6:191–200. https://doi.org/10.1016/s1472-6483(10)61710-5 PMID: 12675999
13. Isachenko V, Isachenko E, Petrunkina AM, Sanchez R. Human spermatozoa vitrified in the absence of permeable cryoprotectants: birth of two healthy babies. Reprod Fertil Dev. 2012; 24:323–26. https://doi.org/10.1071/RD11061 PMID: 22281078
14. Nawroth F, Isachenko V, Dessole S, Rahimi G, Farina M, Vargiu N et al. Vitrification of human spermatozoa without cryo-protectants. CryoLetters. 2002; 23:93–102. PMID: 12050777
15. Bauchi A, Woods EJ, Critser JK. Cryopreservation and vitrification: recent advances infertility preservation technologies. Expert Rev Med Devices. 2008; 5:359–70. https://doi.org/10.1586/17434440.5.3.359 PMID: 18452386
16. Katkov I. Kinetic vitrification of spermatozoa of vertebrates: What we can learn from nature? In: Katkov II editor. Current Frontiers in Cryobiology. Rijeka, Croatia. 2012; 3–40.
17. Sánchez R, Risopatrón J, Schulz M, Villegas J, Isachenko V, Kreinberg R. et al. Canine sperm vitrification with sucrose: effect on sperm function. Andrologia. 2011; 43:233–41. https://doi.org/10.1111/j.1439-0272.2010.01054.x PMID: 21486402
18. Merino O, Sánchez R, Risopatrón J, Isachenko E, Katkov II, Figueroa E, et al. Cryoprotectant-free vitrification of fish (*Oncorhynchus mykiss*) spermatozoa: First report. Andrologia. 2012; 44:390–395. https://doi.org/10.1111/j.1439-0272.2011.01196.x PMID: 21806657
19. Pradiee J, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, Guerra R, et al. Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. Andrologia. 2017; 49:1–7.
20. Shaw JM, Jones GM. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Human Reproduction Update. 2003; 9:583–605. https://doi.org/10.1093/humupd/dmg041 PMID: 14714593
21. Isachenko E, Isachenko V, Weiss JM, Kreienberg R, Katkov II, Schulz M, et al. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction. 2008; 136:167–173. https://doi.org/10.1530/REP-07-0463 PMID: 18483075
22. Caturla-Sánchez E, Sánchez-Calabuig MJ, Pérez-Gutiérrez JF, Cerdeira J, Castaño C, Santiago-Moreno J. Vitrification of dog spermatozoa: Effects of two cryoprotectants (sucrose or trehalose) and two warming procedures. Cryobiology. 2017; 80:126–129. https://doi.org/10.1016/j.cryobiol.2017.11.001 PMID: 29126865
23. Said TM, Gaglani A, Agarwal A. Implication of apoptosis in sperm cryoinjury. Reproductive BioMedicine Online. 2010; 21(4):456–462. https://doi.org/10.1016/j.rbmo.2010.05.011 PMID: 20800544
24. Colás C, Junquera C, Pérez-Pé R, Cebrián-Pérez JA, Muiño-Blanco T. Ultrastructural Study of the Ability of Seminal Plasma Proteins to Protect Ram Spermatozoa Against Cold-shock. Microsc. Res. Tech. 2009; 72:566–572. https://doi.org/10.1002/jemt.20710 PMID: 19322897
25. Gravance CG, Vishwanath R, Pitt C, Garner DL, Casey PJ. Effects of cryopreservation on bull sperm head morphometry. J Androl. 1998; 19:704–709. PMID: 9876021
26. Esteso MC, Fernández-Santos MR, Soler AJ, Garde JJ. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. Cryo-Letters. 2003; 24:261–268. PMID: 12955173
27. Rijsselaere T, Van Soom A, Hoflack G, Maes D, De Kruif A. Automated sperm morphometry and morphology analysis of canine semen by the Hamilton-Thorne analyser. Theriogenology. 2004; 62:1292–306. https://doi.org/10.1016/j.theriogenology.2004.01.005 PMID: 15325556
28. O'Brien E, Esteso MC, Castaño C, Toledano-Diaz A, Bóveda P, Martínez-Fresneda L, et al. Effectiveness of ultra-rapid cryopreservation of sperm from endangered species, examined by morphometric means. Theriogenology. 2019; 129:160–167. https://doi.org/10.1016/j.theriogenology.2019.02.024 PMID: 30852388

29. Auger J, Ronot X, Dadoune JP. Human sperm mitochondrial function related to motility: a flow and image cytometric assessment. J Androl. 1989; 10:439–48. https://doi.org/10.1002/j.1939-4640.1989.tb00135.x PMID: 2621152

30. Sharafi M, Zhandi M, Sharif AA. Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flowcytometric study on post-thawed ram spermatozoa. Cell Tissue Bank. 2015; 16(2):261–9. https://doi.org/10.1007/s10561-014-9458-5 PMID: 24907919

31. Emamverdi M, Zhandi M, Shahneh AZ, Sharafi M, Akhlaghi A, Motlagh, et al. Flow cytometric and microscopic evaluation of post-thawed ram semen cryopreserved in chemically defined home-made or commercial extenders. Animal Production Science. 2015; 55:551–558.

32. Pradiee J, O'Brien E, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, et al. Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (*Rupicapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates. Anim. Reprod. Sci. 2016; 171:121–128. https://doi.org/10.1016/j.anireprosci.2016.06.007 PMID: 27346588

33. Santiago-Moreno J, Castaño C, Toledano-Díaz A, Esteso MC, López-Sebastián A, Guerra R, et al. Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. Theriogenology. 2013; 79:383–391. https://doi.org/10.1016/j.theriogenology.2012.10.011 PMID: 23158213

34. Esteso MC, Rodríguez E, Toledano-Díaz A, Castaño C, Pradiee J, López-Sebastián A. et al. Descriptive analysis of sperm head morphometry in Iberian ibex (*Capra pyrenaica*): Optimum sampling procedure and. Anim. Reprod. Sci. 2015; 155:1–8. https://doi.org/10.1016/j.anireprosci.2015.01.007

35. Forero-González RA, Celeghini ECC, Raphael CF, Andrade AFC, Bressan FF, Arruda RP. Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia. 2012; 44:154–159. https://doi.org/10.1111/j.1439-0272.2010.01154.x PMID: 22506813

36. Chang BS, Randall CS. Use of subambient thermal analysis to optimise protein lyophilisation, Cryobiology. 1992; 29:632–656.

37. Lopera-Vásquez R, Hamdi M, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P, Calle A, et al. Extracellular Vesicles from BOEC in In Vitro Embryo Development and Quality. PLoS ONE. 2016; 11(2).

38. Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. The Journal of Experimental Zoology. 1993; 265:432–437. https://doi.org/10.1002/jez.1402650413 PMID: 8463792

39. Constantin JG, Schneider M and Corti HR. Glass Transition Temperature of Saccharide Aqueous Solutions Estimated with the Free Volume/Percolation Model. J Phys Chem B. 2016; 120(22):5047–55. https://doi.org/10.1021/acs.jpcb.6b01841 PMID: 27176640

40. Angell CA, Oguni M and Sichina WJ. Heat capacity of water at extremes of supercooling and superheating. J Phys Chem. 1982; 86:998–1002.

41. Umrath W. Calculation of the freeze-drying time for electron-microscopical preparations. Mikroskopie. 1983; 40:9–34. PMID: 6343916

42. Teixeira AS, González-Benito ME and Molina-García AD. Tissue and cytoplasm vitrification in cryopreservation monitored by low temperature scanning electron microscopy (cryo-SEM). In: Méndez-Vilas A (ed) Current microscopy contributions to advances in science and technology. Formatex, Badajoz, 2012; 872–879.

43. Teixeira AS, González-Benito ME and Molina-García AD. Determination of glassy state by cryo-SEM and DSC in cryopreservation of mint shoot tips by encapsulation–dehydration. Plant Cell Tiss Organ Cult. 2014; 119:269–280.

44. Marco-Jiménez F, Casares-Crespo L and Vicente JS. Porcine oocyte vitrification in optimized low toxicity solution with open pulled straws. Zygote. 2012; 204–212. https://doi.org/10.1017/S0967199412000524 PMID: 23102007

45. Morris GJ. Rapidly cooled human sperm: no evidence of intracellular ice formation. Human Reproduction. 2006; 21:2075–2083. https://doi.org/10.1093/humrep/del116 PMID: 16613884

46. Morris GJ, Faszer K, Green JE, Draper DE, Grout BWW, Fonseca F. Rapidly cooled horse spermatozoa: Loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. Theriogenology. 2007; 68:804–812. https://doi.org/10.1016/j.theriogenology.2007.06.009 PMID: 17645937

47. Ekwall H. Cryo-scanning electron microscopy (Cryo-SEM) of boar semen frozen in medium-straws and MiniFlatPacks. 2007; 67:1463–1472.

48. Isachenko V, Isachenko E, Katkov I, Montag M, Dessole S, Nawroth F, et al. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapour: effect on motility, DNA

integrity and fertilization ability. Biol Reprod. 2004; 71:1167–73. https://doi.org/10.1095/biolreprod.104.028811 PMID: 15175233

49. Cook KLM, Hartel RW. Ice Crystallization in Ice Cream Production. Compr. Rev. food Sci. food Saf. 2010; 9: 213–222.

50. Caldwell KB, Goff HD, Stanley DW. A Low-Temperature Scanning Electron Microscopy Study of Ice Cream. II. Influence of Selected Ingredients and Processes. Food Structure. 1992; 11:11–23.

51. Aboagla EM, Terada T. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogeniology. 2004; 62:1160–1172.

52. Dubochet J. The Physics of Rapid Cooling and Its Implications for Cryoimmobilization of Cells. Methods Cell Biol. 2007; 79:7–21. https://doi.org/10.1016/S0091-679X(06)79001-X PMID: 17327150

53. Courtens JL, Paquignon M. Ultrastructure of fresh, frozen and frozen-thawed spermatozoa of the boar in: L.A. Johnson, K. Larsson (Eds.), Proceedings of the First International Conference on Deep Freezing of Boar Semen, Swedish University of Agricultural Sciences. 1985; 61–87.

54. Sherman JK, Liu KC. Ultrastructure before freezing, while frozen, and after thawing in assessing cryoinjury of mouse epididymal spermatozoa. Cryobiology. 1988; 19:503–510.

55. Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. International Journal of Andrology. 2012; 35:109–124. https://doi.org/10.1111/j.1365-2605.2011.01218.x PMID: 21950496

56. Somanath PR, Gandhi KK. Isolation and partial characterisation of the plasma and outer acrosomal membranes of goat spermatozoa. Small Ruminant Research. 2004; 53:67–74.

57. Krogenaes A, Berg KA, Hafne AL, Engeland E. Membrane Alterations in Bull Spermatozoa after Freezing and Thawing and after In Vitro Fertilization. Acta vet scand. 1994; 35:17–26. PMID: 8209818

58. Hofmo PO, Berg KA. Electron Microscopical Studies of Membrane Injuries in Blue Fox Spermatozoa Subjected to the Process of Freezing and Thawing. Cryobiology. 1989; 26:124–131. https://doi.org/10.1016/0011-2240(89)90042-4 PMID: 2707028

59. Oliveira LZ, Hossepian de Lima VFM, Levenhagen MA, dos Santos RM, Assumpção TI, Jacomini JO, et al. Transmission electron microscopy for characterization of acrosomal damage after Percoll gradient centrifugation of cryopreserved bovine spermatozoa. J. Vet. Sci. 2011; 12(3): 267–272. https://doi.org/10.4142/jvs.2011.12.3.267 PMID: 21897100

60. Nath J. Correlative Biochemical and Ultrastructural Studies on the Mechanism of Freezing Damage to Ram Semen. Cryobiology. 1972; 246:240–246.

# *ARTÍCULO II*

## Slow and ultra-rapid freezing protocols for cryopreserving mouflon (*Ovis musimon*) and fallow deer (*Dama dama*) epididymal sperm

*Animal Reproduction Science 2018;192:193-199* (Bóveda et al., 2018)

## Protocolos de congelación lenta y ultra-rápida para la criopreservación de espermatozoides epididimarios de muflón (*Ovis musimon*) y gamo (*Dama dama*)

## RESUMEN ARTÍCULO II

Este estudio ha examinado la efectividad de dos métodos para la criopreservación de espermatozoides epididimarios obtenidos *post-mortem* – congelación convencional lenta empleando un tiempo de equilibrado corto con glicerol, y congelación ultra-rápida – de dos especies de rumiantes silvestres *Ovis musimon* (muflón) y *Dama dama* (gamo). Para la congelación convencional de muestras espermáticas de gamo se ha usado un medio compuesto por Tris-ácido cítrico-glucosa (TCG) + 12% de yema de huevo, mientras que para muestras espermáticas de muflón se ha usado un medio compuesto por Tes-Tris-glucosa (TEST) + 6% de yema de huevo. El glicerol ha sido añadido al final para conseguir una concentración del 5% en ambos medios. Los mismos diluyentes han sido usados para la congelación ultra-rápida pero reemplazando el glicerol por 100mM de sucrosa. Las variables espermáticas (motilidad, viabilidad, integridad de acrosoma, integridad de membrana y morfoanomalías) han sido analizadas antes y después de la criopreservación. Aunque los valores han sido, en general, mejores en las muestras espermáticas criopreservadas mediante la congelación convencional, la motilidad total (38,40 ± 4,44% en muflón y 31,25 ± 3,37% en gamo) y el total de espermatozoides vivos (47,19 ± 5,18% en muflón y 43,13 ± 2,43% en gamo) ha sido aceptable para los espermatozoides congelados de manera ultra-rápida. Independientemente del método de criopreservación, la integridad de membrana, la integridad del acrosoma y el porcentaje de espermatozoides muertos y muertos con el acrosoma dañado ha sido mejor para criopreservación de espermatozoides de muflón en comparación con los espermatozoides de gamo ($P<0,05$). A pesar de que la congelación ultra-rápida genera mayor daño en las variables espermáticas que la congelación convencional, podría ser un procedimiento alternativo para la criopreservación de espermatozoides epididimarios en estas especies en condiciones de campo, ya que se trata de una técnica simple que no requiere de un equipo sofisticado ni de una excesiva cualificación técnica.

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# Slow and ultra-rapid freezing protocols for cryopreserving mouflon (*Ovis musimon*) and fallow deer (*Dama dama*) epididymal sperm

P. Bóveda[a], M.C. Esteso[a], C. Castaño[a], A. Toledano-Díaz[a], A. López-Sebastián[a], A. Muñiz[a], P. Prieto[b], O. Mejía[c], R. Ungerfeld[d], J. Santiago-Moreno[a,*]

[a] Department of Animal Reproduction, INIA, Madrid, Spain
[b] D.T. Jaén, Consejería de Medio Ambiente y Ordenación del Territorio, Junta de Andalucía, Spain
[c] Centro de Enseñanza, Investigación y Extensión en Producción Ovina, FMVZ-UNAM, Tres Marías, Morelos 62515, Mexico
[d] Departamento de Fisiología, Facultad de Veterinaria, Universidad de la República, Lasplaces 1550, Montevideo 11600, Uruguay

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ABSTRACT

This study examines the effectiveness of two methods for cryopreserving post-mortem epididymal sperm - conventional slow freezing employing a short equilibration time with glycerol, and ultra-rapid freezing - from the wild ruminant species *Ovis musimon* (mouflon) and *Dama dama* (fallow deer). A Tris-citric acid-glucose (TCG) + 12% egg yolk-based medium was used for the conventional slow freezing of the fallow deer sperm, whereas a Tes-Tris-glucose (TEST) + 6% egg yolk-based medium was used for the mouflon sperm. Glycerol was added to a final concentration of 5% to both media. The same diluents were used for ultra-rapid freezing but replacing the glycerol with 100 mM of sucrose. Sperm variables (motility, viability, acrosome integrity, membrane integrity, and morphological abnormalities) were analyzed before and after cryopreservation. Although values were generally better after the thawing of the conventionally cryopreserved sperm, total sperm motility (38.40 ± 4.44% in mouflon and 31.25 ± 3.37% in fallow deer) and total live sperm (47.19 ± 5.18% in mouflon and 43.13 ± 2.43% in fallow deer) were acceptable for the ultra-rapidly cooled sperm. Independent of the cryopreservation method, membrane integrity, acrosome integrity and the percentages of dead sperm and sperms with a damaged acrosome were better for the cryopreserved mouflon sperm than the fallow deer sperm ($P < 0.05$). Despite exerting a more harmful effect on sperm variables than conventional freezing, ultra-rapid freezing may be a useful alternative for the cryopreservation of these species' epididymal sperm in the field, as this simple technique does not require sophisticated equipment and expertise.

## 1. Introduction

The development of new techniques for sperm cryopreservation (e.g. species-specific customization of freezing extenders, freezing at ultra-rapid cooling rates, directional freezing technique) has allowed progress to be made in the banking of genetic resources and improved the conservation of endangered wild species (Sánchez et al., 2012; Prieto et al., 2014). The widespread use of cryopreservation is, however, limited by the difficulties involved in collecting sperm and freezing it under field conditions. In wild ruminant species such as the European mouflon (*Ovis musimon*) and fallow deer (*Dama dama*), sperm cryopreservation is of great interest not only for conservation purposes, but also for improving the stock on hunting reserves. The methods of assisted reproduction commonly

* Corresponding author.
*E-mail address:* moreno@inia.es (J. Santiago-Moreno).

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used in domestic animals (e.g. artificial insemination with frozen-thawed sperm) are currently being applied to game species, allowing the optimization of wild game management, by means of selection of animals with high genetic value, and improving the quality of the trophies. Moreover, genome resource banking may be the only way to guarantee the continued survival of certain species, subspecies or ecotypes (Santiago-Moreno and López-Sebastián, 2010).

Viable epididymal sperm can be collected and cryopreserved from dead and hunter-killed males of several wild species (Soler et al., 2003; Pérez-Garnelo et al., 2004; Santiago-Moreno et al., 2006a). In Iberian ibexes (*Capra pyrenaica*), the cryopreservation of such sperm can be field-adapted through the use of a short equilibration time and a permeant cryoprotectant (Pradiee et al., 2014). Ultra-rapid cooling might also be used in the field since it requires less equipment than conventional freezing, and is much faster, simpler and cheaper (Isachenko, 2003). It was first used successfully for cryopreserving human (Isachenko et al., 2011) and fish (Merino et al., 2012) sperm, and more recently it has been used to preserve ejaculated Iberian ibex (Pradiee et al., 2015) and European mouflon (Pradiee et al., 2017) sperm. The key to success when using this technique with wild ruminant sperm is to add only moderate concentrations of sucrose (about 100 mM) to the extender, and to use rapid thawing rates when later thawing the sperm (Pradiee et al., 2015, 2017). Both shortening the prefreezing equilibration time with glycerol and ultra-rapid cooling rates have been used with ejaculated mouflon sperm, but with only very moderate success (Pradiee et al., 2016, 2017). Epididymal sperm from some studied species, such as sheep (García-Álvarez et al., 2009) and ibex (Pradiee et al., 2014, 2016), were found to be more cryoresistant than sperm obtained by electro-ejaculation. This advantage was also seen when comparing epididymal ram sperm with normally ejaculated sperm (Woelders et al. 2012). Therefore, ultra-rapid freezing of epididymal mouflon sperm might give good results.

The aim of the present work was to examine the effect of two freezing protocols - conventional freezing with a short equilibration time with glycerol, and ultra-rapid freezing - on the quality of frozen/thawed epididymal sperm from mouflon and fallow deer. The literature contains no information on the use of short equilibration times or ultra-rapid freezing applied to ejaculated or epididymal sperm from fallow deer, or to mouflon epididymal sperm.

## 2. Materials and methods

### 2.1. Animals and sperm collection

Testes were collected during the rutting season (December of 2014 and February of 2015) from dead mature mouflons (n = 16) and fallow deer (n = 8). All animals had been legally hunted in their natural habitat in the Andalusian hunting reserve of Cazorla and Segura (Jaén, Spain), in accordance with the reserve's harvesting plan. The latter plan followed Spain's 'Harvest Regulation, Forest and Wild Animal Law 8/2003' issued by the Andalusian Regional Government, which adheres to European Union regulations.

### 2.2. Sperm collection

The sperm collected from the left and right epididymides was cryopreserved differently: classic slow freezing for the sperm from the left epididymis with a short equilibration time with glycerol (Pradiee et al., 2014), and ultra-rapid freezing for sperm collected from the right epididymis. Sperm collected from the left epididymis (for conventional freezing) of the fallow deer was collected by retrograde flushing with 1 ml of TCG (Tris (313.7 mM), citric acid (104.7 mM), glucose (30.3 mM)) + 12% egg yolk (vol/vol) at 5 °C (Santiago-Moreno et al., 2009), while the corresponding sperm from the mouflon was collected in the same way using TTG (Tes (210.6 mM), Tris (95.8 mM), glucose (10.1 mM) + 6% egg yolk (vol/vol)). Sperm collected from the right epididymis (for ultra-rapid cryopreservation) was flushed out using the same method and extenders for each species, but including additionally 100 mM of sucrose in the flushing solution. These flushing agents were prepared using reagent-grade chemicals purchased from Panreac Química S.A. (Barcelona, Spain) and the Sigma Chemical Co. (St. Louis, Missouri, USA).

### 2.3. Sperm assessment

Total sperm concentration in the collected samples was calculated before freezing using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility variables, sperm morphology, acrosome and plasma membrane integrity were assessed for each sample both prior to freezing and after later thawing. The percentage of motile spermatozoa and the quality of motility were evaluated subjectively using a phase contrast microscope (Zeiss, Oberkochen, Germany). The quality of sperm movement was scored on a scale from 0 (lowest) to 5 (highest). Sperm motility was also evaluated objectively using a computer-aided sperm analysis system (CASA) (SCA, Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capability. For this, sperm samples were diluted with the same extender used for flushing the sperm from the epididymis and 3 µL drops were placed on a Leja eight-chamber slide (Leja Products B.V., Nieuw Vennep, The Netherlands). A minimum of three fields and 500 sperm tracks were assessed to determine the percentage of immotile sperm, sperm showing non-progressive motility, and sperm showing progressive motility.

Plasma membrane integrity was assessed by nigrosin-eosin staining (Campbell et al., 1956) and the hypo-osmotic swelling test (Jeyendran et al., 1984). The percentages of spermatozoa with a normal acrosomal ridge (NAR; Pursel and Johnson, 1974; Pintado and Pérez-Llano, 1992) and morphological abnormalities (Frank 1950) were assessed by examination of glutaraldehyde-fixed samples under a phase-contrast microscope. Sperm cell morphology was categorized as either normal or as showing coiled tails, bent tails, loose normal heads, broken necks, abnormal heads, mid-piece defects, or proximal or distal cytoplasmic droplets. Spermatozoa with cytoplasmic droplets were considered morphologically normal since these are commonly seen in epididymal sperm cells. Sperm

viability and acrosomal status were also analysed by fluorescence microscopy. These features were simultaneously evaluated using a fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC), as previously described (Soler et al., 2005). Four classes of spermatozoa can be distinguished with PI/PNA-FITC: 1) live spermatozoa with an intact acrosome (PI-/PNA-FITC-), 2) live spermatozoa with a damaged acrosome (PI-/PNA-FITC+), 3) dead spermatozoa with an intact acrosome (PI+/PNA-FITC-), 4) dead spermatozoa with a damaged acrosome (PI+/PNA-FITC+). Each analysis required the observation of 200 cells.

Finally smears were prepared for morphometric analysis by placing 5 µl sperm samples on the clear end of a slide and then dragging the drop across the slide. For fresh semen smears of both species, a 5 µl aliquot of semen from all right, and all left, sampled epididymides was mixed. The smears were allowed dry at room temperature before staining. Subsequently, samples were stained with Hemacolor and assessed by CASA using the morphometry module of Sperm-Class Analyzer v.5.3.0.1 software (Microptic S.L. Barcelona, Spain) as described by Esteso et al. (2015). A total of 100 sperm heads were analysed per slide.

### 2.4. Sperm cryopreservation

Immediately upon collection, the sperm samples were diluted using 1 mL of the above-stated media (TCG for fallow deer sperm, TEST for mouflon sperm), plus, depending on the experimental group to which samples were assigned, either glycerol (final concentration 5%) (TCG-gly/TEST-gly for conventional slow freezing) or 100 mM sucrose (TCG-100mM sucrose/TEST-100mM for ultra-rapid cryopreservation). All samples were diluted with the same media used for collection, achieving a concentration of $800 \times 10^6$ sperm/mL, and transferred to 15 ml centrifuge tubes (Sterilin, Stone, UK).

Sperm samples used for conventional slow freezing (left epididymis) were cooled for 1 h at 5 °C (Pradiee et al., 2014). After an equilibration period of 15 min, aliquots of samples were loaded into 0.25 mL straws and frozen by placing the samples in the nitrogen vapor 5 cm above the surface of a liquid nitrogen bath for 10 min before plunging them into the liquid nitrogen, providing the following cooling rate: from 5 °C to −35 °C at 40 °C/min, from −35 °C to −65 °C at 17 °C/min, from −65 °C to −85 °C at 3 °C/min, and then transfer into liquid nitrogen to cool to −196 °C. The procedure was standardized to ensure such cooling and checked using a Ventix® K/J/T thermometer (Ventix, China) equipped with a probe resistant to freezing (Esteso et al., 2018). After a storage period (no more than a year), the straws were thawed in a water bath at 37 °C for 30 s and the contents emptied into 1.5 mL Eppendorf microcentrifuge tubes (Eppendorf Ibérica SLU, Madrid, Spain), and incubated for 5 min at the same temperature (Pradiee et al., 2014, 2015). Sperm quality was then analysed as described above.

Sperm samples for ultra-rapid cryopreservation (right epididymis) were placed in 15 mL centrifuge tubes and cooled for 30 min at 5 °C. Samples were then pipetted and plunged drop-by-drop (about 50 µL per drop) directly into liquid nitrogen (Pradiee et al., 2015). After storage, the frozen pellets were quickly thawed placing them on a DPP70 thermoregulated conical hotplate (INIA, Madrid, Spain) set at 60–65 °C. The thawed sperm fell into a glass beaker within 2–5 s. The resultant samples were maintained at 37 °C during the assessment of sperm quality (Pradiee et al., 2015).

### 2.5. Statistical analysis

The values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilks test, were arcsine-transformed before analysis. The *t*-test for matched pairs was used to compare the within-species effect of the cryopreservation method on sperm variables. The same test was used to examine within-method changes in sperm quality caused by cryopreservation, comparing values immediately before freezing and immediately after thawing.

The influence of species on fresh sperm head morphometric values was examined by ANOVA, followed by a post-hoc Tukey Test when significant differences were detected. The statistical model was as follows: $x_{ij} = m + A_i + e_{ij}$, where $x_{ij}$ = the accuracy of the assay, m = the overall mean of measurable sperm head morphometric variables, $A_i$ = the effect of species (i = 1–2), and $e_{ij}$ = the residual (j = 1–24). Differences in morphometric sperm variables after cryopreservation were assessed by two-way ANOVA followed by a post hoc Tukey Test for multiple comparisons following the statistical model: $x_{ijk} = m + A_i + B_j + AB_{ij} + e_{ijk}$, where $x_{ij}$ = the measured sperm head variable, m = the overall mean of variable x, $A_i$ = the effect of the cryopreservation method (i = 1–2), $B_j$ = the effect of species (j = 1–2), $AB_{ij}$ = the interaction between the cryopreservation method and species, and $e_{ijk}$ = the residual (j = 1–24). Data were expressed as mean ± SE. Where applicable, significance was set at $p < 0.05$. All statistical analyses were performed using STATISTICA software for Windows v.12.0 (StatSoft Inc., Tulsa, OK, USA).

## 3. Results

In mouflons, the post-thaw sperm quality values after conventional freezing-thawing were not significantly different from the pre-freeze values for the percentage of motile sperm, the quality of movement, or the percentage of dead sperm with an intact acrosome; for all the other variables measured, post thaw values were significantly different ($P < 0.05$) (Table 1). The values after ultra-rapid freezing-thawing were not significantly different from the pre-freeze values for the percentage of sperm with morphological abnormalities; for all the other parameters measures, post-ultra-rapid thaw values were significantly different ($P < 0.05$) (Table 1). Significant differences ($P < 0.05$) were recorded between the two treatments in terms of the number of immotile sperm, the number showing non-progressive and progressive motility, and membrane functional integrity, always in favour of the conventional freezing procedure (Table 1).

In fallow deer, the post-thaw sperm values after conventional freezing-thawing were not significantly different from the pre-freeze

**Table 1**
Quality of fresh and thawed mouflon epididymal spermatozoa subjected to slow conventional freezing or ultra-rapid cryopreservation. Values are mean ± SE.

| Sperm variables | Pre-conventional freezing | Pre-ultra-rapid cryopreservation | Post-thawing (conventional freezing) | Post-thawing (ultra-rapid cryopreservation) |
|---|---|---|---|---|
| Motile sperm (%) | 61.25 ± 5.45 | 60.62 ± 5.34* | 50.33 ± 4.26 | 38.44 ± 4.44* |
| Quality of motility (0-5) | 2.81 ± 0.22 | 2.78 ± 0.22* | 2.27 ± 0.17 | 2.09 ± 0.20* |
| Immotile sperm (%) | 20.76 ± 5.89* | 21.72 ± 5.83* | 45.09 ± 7.45a* | 79.73 ± 2.53b* |
| Non-progressive motility (%) | 48.11 ± 3.83* | 48.40 ± 3.77* | 32.29 ± 4.75a* | 12.28 ± 1.57b* |
| Progressive motility (%) | 31.18 ± 4.23* | 29.94 ± 3.87* | 22.62 ± 3.72a* | 7.99 ± 1.25b* |
| NAR (%) | 91.25 ± 1.70* | 91.31 ± 1.70* | 55.60 ± 4.14* | 45.56 ± 3.09* |
| HOST (%) | 65.25 ± 5.96* | 64.87 ± 5.87* | 36.80 ± 3.49a* | 17.69 ± 1.80b* |
| Viable (E/N) (%) | 79.50 ± 2.18* | 79.25 ± 2.18* | 43.80 ± 5.29* | 34.25 ± 2.17* |
| Dead sperm/intact acrosome (%) | 29.50 ± 2.91 | 29.62 ± 2.86 | 35.80 ± 4.41 | 32.44 ± 4.87 |
| Dead sperm/damaged acrosome (%) | 0.19 ± 0.01* | 0.25 ± 0.11* | 15.73 ± 3.14* | 20.38 ± 2.61* |
| Live sperm/intact acrosome (%) | 70.25 ± 2.92* | 70.06 ± 2.85* | 35.13 ± 5.39* | 38.06 ± 4.13* |
| Live sperm/damaged acrosome (%) | 0.00 ± 0.00* | 0.00 ± 0.00* | 13.33 ± 2.50* | 9.13 ± 2.23* |
| Total live sperm (%) | 70.25 ± 2.92* | 70.06 ± 2.85* | 48.47 ± 6.29* | 47.19 ± 5.18* |
| Total dead sperm (%) | 29.50 ± 2.91* | 29.88 ± 2.84* | 51.53 ± 6.29* | 52.81 ± 5.18* |
| Morphological abnormalities (%) | 14.88 ± 3.16* | 15.19 ± 3.15 | 27.63 ± 2.97* | 20 ± 3.28 |

Different lower case letters (a, b) indicate significant differences ($P < 0.05$) between cryopreservation methods. Asterisks indicate significant differences ($P < 0.05$) between pre-conventional freezing *vs* post-thawing (conventional freezing), and pre-ultra-rapid cryopreservation *vs* post-thawing (ultra-rapid cryopreservation). Motile sperm (%) and the quality of motility were evaluated subjectively using a phase contrast microscope. Immotile sperm (%), non-progressive motility (%), progressive motility (%) were evaluated by CASA. NAR: Acrosome ridge integrity; HOST: hypo-osmotic test for membrane functional integrity; Viable (E/N): Sperm viability according eosin-nigrosin staining.

values for the percentage of motile sperm, the quality of movement, nor the percentage of sperm showing progressive motility; for the remaining variables, post thaw values were significantly different ($P < 0.05$) (Table 2). The values after ultra-rapid freezing-thawing were not significantly different from the pre-freeze values for the percentage of sperm with morphological abnormalities; for all the other variables measures, post-ultra-rapid thaw values were significantly different ($P < 0.05$) (Table 2). Significant differences ($P < 0.05$) were recorded between the two treatments in terms of the number of immotile sperm, the number showing non-progressive and progressive motility, acrosome ridge integrity, membrane functional integrity, sperm viability according to eosin-nigrosin staining, and the percentage of dead sperm with a damaged acrosome, always in favour of the conventional freezing procedure (Table 2).

The mouflon sperm cells were larger than those of the fallow deer ($P < 0.05$). Post-thaw sperm heads were smaller compared with pre-freeze for both freezing methods, with significant differences between pre-freeze and post-thaw for all head measures of fallow deer and mouflon sperm except for mouflon head width. The reduction in size was significantly stronger ($P < 0.05$) in ultra-rapid frozen semen than in slow-frozen semen, for all head measures of fallow deer and mouflon sperm except for mouflon head width and area (Tables 3 and 4).

**Table 2**
Quality of fresh and thawed fallow deer epididymal spermatozoa subjected to slow conventional freezing or ultra-rapid cryopreservation. Values are mean ± SE.

| Sperm variables | Pre-conventional freezing | Pre-ultra-rapid cryopreservation | Post-thawing (conventional freezing) | Post-thawing (ultra-rapid cryopreservation) |
|---|---|---|---|---|
| Motile sperm (%) | 53.13 ± 6.12 | 51.86 ± 5.74* | 45 ± 5.18 | 31.25 ± 3.37* |
| Quality of motility (0-5) | 2.69 ± 0.28 | 2.56 ± 0.32* | 2.06 ± 0.15 | 1.68 ± 0.19* |
| Immotile sperm (%) | 11.96 ± 2.71* | 11.71 ± 2.70* | 43.80 ± 4.80a* | 84.91 ± 1.18b* |
| Non-progressive motility (%) | 69.33 ± 3.28* | 71.40 ± 3.00* | 31.70 ± 2.82a* | 11.60 ± 0.95b* |
| Progressive motility (%) | 18.68 ± 2.79 | 16.86 ± 2.58* | 24.49 ± 2.53a | 3.51 ± 0.95b* |
| NAR (%) | 84.63 ± 2.09* | 86.25 ± 2.30* | 49.50 ± 3.36a* | 37 ± 3.03b* |
| HOST (%) | 51.63 ± 14.77* | 51 ± 14.59* | 27.25 ± 3.18a* | 5.16 ± 1.33b* |
| Viable (E/N) (%) | 90.88 ± 1.90* | 89.63 ± 2.10* | 51.25 ± 3.73a* | 36.25 ± 2.04b* |
| Dead sperm/intact acrosome (%) | 12.63 ± 1.95* | 14.38 ± 2.56* | 36.75 ± 4.03a* | 25.36 ± 4.13b* |
| Dead sperm/damaged acrosome (%) | 0.75 ± 0.75* | 0.63 ± 0.63* | 19 ± 3.30a* | 31.5 ± 4.46b* |
| Live sperm/intact acrosome (%) | 86.63 ± 2.05* | 85.00 ± 3.00* | 37.75 ± 4.71* | 38.86 ± 2.89* |
| Live sperm/damaged acrosome (%) | 0.00 ± 0.00* | 0.00 ± 0.00* | 6.5 ± 2.49* | 4.25 ± 1.22* |
| Total live sperm (%) | 86.63 ± 2.05* | 85.00 ± 3.00* | 43.88 ± 5.98* | 43.13 ± 2.43* |
| Total dead sperm (%) | 13.38 ± 2.05* | 15.00 ± 3.00* | 56.13 ± 5.98* | 56.88 ± 2.43* |
| Morphological abnormalities (%) | 11.88 ± 2.33* | 10.63 ± 2.49 | 26.38 ± 3.56a* | 17.5 ± 3.01b |

Different lower case letters (a, b) indicate significant differences ($P < 0.05$) between cryopreservation methods. Asterisks indicate significant differences ($P < 0.05$) between pre-conventional freezing *vs* post-thawing (conventional freezing), and pre-ultra-rapid cryopreservation *vs* post-thawing (ultra-rapid cryopreservation). Motile sperm (%) and the quality of motility were evaluated subjectively using a phase contrast microscope. Immotile sperm (%), non-progressive motility (%), progressive motility (%) were evaluated by CASA. NAR: Acrosome ridge integrity; HOST: hypo-osmotic test for membrane functional integrity; Viable (E/N): Sperm viability according eosin-nigrosin staining.

**Table 3**
Morphometric values (mean ± SE) for fresh and thawed mouflon epididymal spermatozoa subjected to slow conventional freezing or ultra-rapid cryopreservation.

| Morphometric variables | Fresh | Post-thawing (conventional freezing) | Post-thawing (ultra-rapid cryopreservation) |
|---|---|---|---|
| Length (μm) | 8.88 ± 0.01$^{*}$ | 8.80 ± 0.01$^{a*}$ | 8.66 ± 0.01$^{b*}$ |
| Width (μm) | 4.81 ± 0.00$^{*}$ | 4.73 ± 0.01$^{a*}$ | 4.80 ± 0.01 $^{b}$ |
| Area (μm$^2$) | 35.19 ± 0.05$^{*}$ | 34.34 ± 0.1$^{*}$ | 34.30 ± 0.08$^{*}$ |
| Perimeter (μm) | 23.47 ± 0.02$^{*}$ | 23.23 ± 0.03$^{a*}$ | 23.05 ± 0.03 $^{b*}$ |

Different lower case letters (a, b) indicate significant differences (P < 0.05) between cryopreservation methods. Asterisks indicate significant differences (P < 0.05) between fresh samples and post-thawing.

**Table 4**
Morphometric values (mean ± SE) for fresh and thawed fallow deer epididymal spermatozoa subjected to slow conventional freezing or ultra-rapid cryopreservation.

| Morphometric variables | Fresh | Post-thawing (conventional freezing) | Post-thawing (ultra-rapid cryopreservation) |
|---|---|---|---|
| Length (μm) | 7.87 ± 0.01$^{*}$ | 7.86 ± 0.01$^{a*}$ | 7.73 ± 0.02 $^{b*}$ |
| Width (μm) | 4.48 ± 0.00$^{*}$ | 4.40 ± 0.01$^{a*}$ | 4.35 ± 0.01 $^{b*}$ |
| Area (μm$^2$) | 29.17 ± 0.05$^{*}$ | 28.61 ± 0.08$^{a*}$ | 27.90 ± 0.09 $^{b*}$ |
| Perimeter (μm) | 21.20 ± 0.02$^{*}$ | 21.06 ± 0.03$^{a*}$ | 20.75 ± 0.04 $^{b*}$ |

Different lower case letters (a, b) indicate significant differences (P < 0.05) between cryopreservation methods. Asterisks indicate significant differences (P < 0.05) between fresh samples and post-thawing.

## 4. Discussion

For both species, the conventionally cryopreserved sperm returned better quality results after thawing, although the sperm thawed after ultra-rapid freezing returned acceptable total sperm motility and sperm viability values. Ultra-rapid freezing may therefore provide an alternative to conventional freezing method under field conditions.

When either cryopreservation technique was used, the sperm of both species showed reduced membrane functionality according to the hypo-osmotic swelling test, reduced acrosome integrity, and increased numbers of dead sperms with a damaged acrosome. However, post-thaw the percentages of NAR and HOST appeared to be lower in fallow deer than in mouflon, suggesting that the two species differ in their sperm cryoresistance, albeit that the used freezing media were a confounding factor.

The success of cryopreservation by both the slow and ultra-rapid freezing procedures is highly dependent on the buffer system and concentrations of cryoprotectant used (Santiago-Moreno et al., 2006b; Pradiee et al., 2017). Differences between the species in response to certain media components may be due to species-specific properties of sperm membrane fluidity, the cholesterol/phospholipid ratio, and/or osmotic tolerance. For example, previous studies in rams (*Ovis aries*), aoudad (*Ammotragus lervia*) and mouflon have highlighted the benefits of using zwitterion buffers such as TES, HEPES or PIPES (Molinia et al., 1994; Santiago-Moreno et al., 2013; Pradiee et al., 2016). However, some of these seem to be harmful to ibex sperm (Santiago-Moreno et al., 2009). In the present study, the most appropriate extender composition for each species was chosen. Thus, taking into account the results of previous studies in mouflon (Pradiee et al., 2017), a TEST extender including the zwitterion buffer TES was used for this species. For fallow deer, and other cervid species, Triladyl (Zomborszky et al., 2005) and Tris-citric acid–glucose based extenders (Mulley et al., 1988) have been successfully used in sperm freezing. Thus, a TCG-based extender was used in the present work.

Data revealed that sometimes the percentages of live sperm according PI, and the percentage of viable sperm according eosin-nigrosin stain didn't match with the percentage of sperm with functional membrane according to hypoosmotic test (HOST). This may be explained because the membrane swelling during HOST is particularly noticeable at the sperm tail; the membranes of sperm tail and sperm head may function independently from each other, and thus tail swelling might not be indicative of normal functional activity of the head membranes (Jeyendran et al., 1984).

The literature contains few reports on the use of ultra-rapid freezing to preserve the sperm of wild ruminants (Pradiee et al., 2015). Given the poor tolerance of sperm from wild ruminants to the osmotic and cytotoxic effects of high concentrations of permeable cryoprotectants, alternative solutions based on the addition of disaccharides have been designed and tested, with 100 mM of sucrose found to be the most effective (Pradiee et al., 2015, 2017). The addition of certain low concentrations of non-permeable cryoprotectants such as albumin, and of osmoprotectants such as disaccharides (mainly sucrose) in the ultra-rapid freezing medium has been reported to improve the cryosurvival of human (Isachenko et al., 2008) and dog sperm (Sánchez et al., 2011).

The cryopreservation of sperm from wild ruminants commonly has to be performed in field laboratories close to where the samples are obtained. Shortening the time required to complete the procedure would be very advantageous under such conditions. The use of ultra-rapid cooling rates has several advantages over conventional slow freezing, including its simplicity and the time required to perform it; only 30 min of equilibration time are needed plus a few seconds in contact with liquid nitrogen. However, the present findings show the frozen/thawed sperm variables of conventionally cryopreserved sperms to be better. Traditionally, long equilibration periods are used for both epididymal and ejaculated sperm. Long exposure to cryoprotectants can, however, be harmful to sperm (Gao et al., 1993; Katkov et al., 1998); reducing these exposure times might, therefore, afford advantages other than shortening the processing time. Although in the majority of wild ruminant species (chamois (*Rupicapra pyrenaica*), ibex, mouflon and

aoudad) ejaculated sperm returns better results after long equilibration periods with glycerol (Pradiee et al., 2016), epididymal sperm of ibexes only required short equilibration times before freezing (Pradiee et al., 2014). In the present work, the conventional slow freezing protocol involved a short equilibration period of just 15 min. This methodology was here used for the first time with epididymal sperm from mouflon and fallow deer and returned successful results. Epididymal sperm seem to be more cryoresistant than ejaculated sperm, with differences in membrane composition (Eddy and O'Brien, 1994; Jones, 1998; Varisli et al., 2009) influencing the equilibration time required (Rath and Niemann, 1997). We have seen evidence that sperm heads of epididymal sperm may be smaller. The head dimensions of both the present mouflon (35.19 $\mu m^2$) and fallow deer (29.17 $\mu m^2$) epididymal sperm were smaller than those reported for ejaculated sperm (39.00 $\mu m^2$ in mouflon (Pradiee et al., 2016), and 29.80 $\mu m^2$ in fallow deer [unpublished]). This could be related with the capacitated status and membrane stability, having influence to better freezability. In addition, a higher osmotic tolerance by epididymal sperm may be expected because the small size of the sperm heads compared to ejaculated sperm. Differences in sperm head sizes may influence sperm water volume as well as glycerol and water fluxes during equilibration times.

In both species the cryopreservation process led to a reduction in sperm head size. This finding agrees with that reported for goat (*Capra hircus*) (Hidalgo et al., 2007), red deer (*Cervus elaphus*) (Esteso, 2006), ibex, aoudad and chamois sperm (Pradiee et al., 2016). Many theories have been proposed to explain this, such as the over-condensation of the sperm chromatin, or the presence of a greater number of sperms with a damaged or lost acrosome, or by the osmotic stress experienced during freezing and thawing causing damage to the cell membrane and cytoskeleton (Gao and Zhou 2012; Thompson et al., 1994; Arruda et al., 2002, Santiago-Moreno et al., 2016). Sperm cryoresistance may in fact be related to the degree of head dimension changes during freezing/thawing; certainly, in giant panda (*Ailuropoda melanoleuca*) sperm, which is highly resistant even to repeated freezing/thawing, the sperm head size changes very little (Santiago-Moreno et al., 2016). The present work shows that, in fallow deer, the sperm head area was smaller after ultra-rapid cryopreservation than after conventional freezing, suggesting greater sensitivity to the latter method. Mouflon sperms were more resistant to ultra-rapid freezing, and the sperm head area after thawing was similar to that recorded for sperms processed by the conventional method.

In conclusion, ultra-rapid freezing may be a useful alternative for cryopreserving both mouflon and fallow deer sperm, although it is more harmful than conventional freezing with respect to sperm motility variables. The data suggest that the intensity of sperm head dimension changes during cryopreservation may reflect cryoresistance.

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## References

Arruda, R.P., Gravance, C.G., Garcia, A.R., Liu, I.K.M., 2002. Effects of extenders and cryoprotectants on stallion sperm head morphology. Theriogenology 58, 253–256.
Campbell, R.C., Dott, H.M., Glover, T.D., 1956. Nigrosin eosin as a stain for differentiating live and dead spermatozoa. J. Agric. Sci. 48, 1–8.
Eddy, E.M., O'Brien, D.A., 1994. In: In: Knobil, E., Neill, J. (Eds.), The Physiology of Reproduction, vol. 1. pp. 29–77.
Esteso, M.C., 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples. J. Androl. 27, 662–670.
Esteso, M.C., Rodríguez, E., Toledano-Díaz, A., Castaño, C., Pradiee, J., López-Sebastián, A., et al., 2015. Descriptive analysis of sperm head morphometry in Iberian ibex (*Capra pyrenaica*): optimum sampling procedure and staining methods using Sperm-Class Analyzer®. Anim. Reprod. Sci. 155, 42–49.
Esteso, M.C., Toledano-Díaz, A., Castaño, C., Pradiee, J., Lopez-Sebastián, A., Santiago-Moreno, J., 2018. Effect of two cooling protocols on the post-thaw characteristics of Iberian ibex sperms. Cryobiology 80, 12–17.
Frank, H., 1950. Microscopic examination of semen. Artificial Insemination in Livestock Breeding. Pathological Division. Bureau of Animal Industry, Agricultural Research Administration. Circular 567 U.S. Department of Agriculture pp. 12-16.
Gao, D.Y., Ashworth, E., Watson, P.F., Kleinhans, F.W., Mazur, P., Critser, J.K., 1993. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. Biol. Reprod. 49, 112–123.
Gao, D., Zhou, X., 2012. Prevention of lethal osmotic injury to cells during addition and removal of cryoprotectibve agents: theory and technology. In: Katkov, I.I. (Ed.), Current Frontiers in Cryobiology, Rijeka, Croatia, pp. 101–136.
García-Álvarez, O., Maroto-Morales, A., Martínez-Pastor, F., Garde, J.J., Ramón, M., Fernández-Santos, M.R., et al., 2009. Sperm characteristics and in vitro fertilization ability of thawed spermatozoa from Black Manchega ram: Electroejaculation and postmortem collection. Theriogenology 72, 160–168.
Hidalgo, M., Rodríguez, I., Dorado, J.M., 2007. The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. Anim. Reprod. Sci. 100, 61–72.
Isachenko, E., 2003. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reprod. Biomed. 6 (2), 191–200 Online.
Isachenko, E., Isachenko, V., Weiss, J.M., Kreienberg, R., Katkov, I.I., Schulz, M., et al., 2008. Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction 136, 167–173.
Isachenko, V., Maettner, R., Petrunkina, A.M., Mallmann, P., Rahimi, G., Sterzik, K., et al., 2011. Cryoprotectant-free vitrification of human spermatozoa in large (up to 0.5 ml) volume: a novel technology. Clin. Lab. 57, 643–650.
Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70, 219–228.
Jones, R., 1998. Plasma membrane structure and remodelling during sperm maturation in the epididymis. J. Reprod. Fertil. Suppl. 53, 73–84.
Katkov, I.I., Katkova, N., Critser, J.K., Mazur, P., 1998. Mouse spermatozoa in high concentrations of glycerol: chemical toxicity vs osmotic shock at normal and reduced oxygen concentrations. Cryobiology 37, 325–338.
Merino, O., Sánchez, R., Risopatrón, J., Isachenko, E., Katkov, I.I., Figueroa, E., et al., 2012. Cryoprotectant-free vitrification of fish (*Oncorhynchus mykiss*)

spermatozoa: first report. Andrologia 44, 390–395.
Molinia, F.C., Evans, G., Maxwell, W.M.C., 1994. Incorporation of penetrating cryoprotectants in diluents for pellet-freezing ram spermatozoa. Theriogenology 42, 849–858.
Mulley, R.C., Moore, N.W., English, A.W., 1988. Successful uterine insemination of fallow deer with fresh and frozen semen. Theriogenology 29, 1149–1153.
Pérez-Garnelo, S.S., Garde, J., Pintado, B., Borque, C., Talavera, C., Delclaux, M., et al., 2004. Characteristics and in vitro fertilizing ability of giant panda (*Ailuropoda melanoleuca*) frozen-thawed epididymal spermatozoa obtained 4 hours postmortem: a case report. Zoo. Biol. 23, 279–285.
Pintado, B., Pérez-Llano, B., 1992. Effect of glutaraldehyde concentration and fixative temperature on the number of spermatozoa with normal acrosomes in goat semen. Theriogenology 38, 527–533.
Pradiee, J., Esteso, M.C., Castaño, C., Toledano-Díaz, A., López-Sebastián, A., Santiago-Moreno, J., 2014. Cryopreservation of epididymal sperm from ibexes (*Capra pyrenaica*) using short equilibration time with glycerol. Theriogenology 82, 525–528.
Pradiee, J., Esteso, M.C., Lopez-Sebastián, A., Toledano-Díaz, A., Castaño, C., Carrizosa, J.A., et al., 2015. Successful ultra-rapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa. Theriogenology 84, 1513–1522.
Pradiee, J., O'Brien, E., Esteso, M.C., Castaño, C., Toledano-Díaz, A., Lopez-Sebastián, A., et al., 2016. Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (*Rupicapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates. Anim. Reprod. Sci. 171, 121–128.
Pradiee, J., Esteso, M.C., Castaño, C., Toledano-Díaz, A., Lopez-Sebastián, A., Guerra, R., et al., 2017. Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. Andrologia 49, 1–7.
Prieto, M.T., Sanchez-Calabuig, M.J., Hildebrandt, T.B., Santiago-Moreno, J., Saragusty, J., 2014. Sperm cryopreservation in wild animals. Eur. J. Wildl. Res. 60, 851–864.
Pursel, V., Johnson, L., 1974. Glutaraldehyde fixation of boar spermatozoa for acrosome evaluation. Theriogenology 1, 63–68.
Rath, D., Niemann, H., 1997. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology 47, 785–793.
Sánchez, R., Risopatrón, J., Schulz, M., Villegas, J., Isachenko, V., Kreinberg, R., et al., 2011. Canine sperm vitrification with sucrose: effect on sperm function. Andrologia 43, 233–241.
Sánchez, R., Risopatrón, J., Schulz, M., Villegas, J.V., Isachenko, V., Isachenko, E., 2012. Vitrified sperm banks: the new aseptic technique for human spermatozoa allows cryopreservation at -86°C. Andrologia 44, 433–435.
Santiago-Moreno, J., Toledano-Díaz, A., Pulido-Pastor, A., Gómez-Brunet, A., López-Sebastián, A., 2006a. Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death. Theriogenology 66, 283–291.
Santiago-Moreno, J., Toledano-Díaz, A., Pulido-Pastor, A., Dorado, J., Gómez-Brunet, A., López-Sebastián, A., 2006b. Effect of egg yolk concentration on cryopreserving Spanish ibex (*Capra pyrenaica*) epididymal spermatozoa. Theriogenology 66, 1219–1226.
Santiago-Moreno, J., Astorga, R.J., Luque, I., Coloma, M.A., Toledano-Díaz, A., Pulido-Pastor, A., et al., 2009. Influence of recovery method and microbial contamination on the response to freezing-thawing in ibex (*Capra pyrenaica*) epididymal spermatozoa. Cryobiology 59, 357–362.
Santiago-Moreno, J., López-Sebastián, A., 2010. Ungulados silvestres de España : biología y tecnologías reproductivas para su conservación y aprovechamiento cinegético Monografías INIA: Serie Medioambiental no. 2.
Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Esteso, M.C., López-Sebastián, A., Guerra, R., et al., 2013. Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. Theriogenology 79, 383–391.
Santiago-Moreno, J., Esteso, M.C., Pradiee, J., Castaño, C., Toledano-Díaz, A., O'Brien, E., et al., 2016. Giant panda (*Ailuropoda melanoleuca*) sperm morphometry and function after repeated freezing and thawing. Andrologia 48, 470–474.
Soler, A.J., García, A.J., Fernández-Santos, M.R., Esteso, M.C., Garde, J.J., 2003. Effects of thawing procedure on post-thawed in vitro viability and in vivo fertility of red deer epididymal spermatozoa cryopreserved at -196°C. J. Androl. 24, 746–756.
Soler, A.J., Esteso, M.C., Fernández-Santos, M.R., Garde, J.J., 2005. Characteristics of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa cryopreserved after storage at 5 °C in the epididymis for several days. Theriogenology 64, 1503–1517.
Thompson, L.A., Brook, P.F., Warren, M.A., Barratt, C.L.R., Cooke, I.D., 1994. A morphometric comparison of the nuclear morphology of fresh and frozen-thawed human zona-bound and unbound sperm. J. Androl. 15, 337–342.
Varisli, O., Uguz, C., Agca, C., Agca, Y., 2009. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. Anim. Reprod. Sci. 110, 256–268.
Woelders, H., Windig, J., Hiemstra, S.J., 2012. How developments in cryobiology, reproductive technologies and conservation genomics could shape gene banking strategies for (farm) animals. Reprod. Dom. Anim. 47 (Suppl. 4), 264–273.
Zomborszky, Z., Nagy, S., Nánássy, L., Szabari, M., Bodó, S., 2005. Experiences in deer sperm cryopreservation under practical conditions — A pilot study. Anim. Reprod. Sci. 90, 185–190.

# ARTÍCULO III

## Influence of circulating testosterone concentration on sperm cryoresistance: The ibex as an experimental model

*Andrology 2021;00:1–12* (Bóveda et al., 2021)

## Influencia de las concentraciones circulantes de testosterona en la criorresistencia espermática: uso del macho montés como modelo experimental

## RESUMEN ARTÍCULO III

Estudios recientes han apuntado que las concentraciones circulantes de testosterona podrían afectar a la capacidad de los espermatozoides a sobrevivir a los procesos de criopreservación. Sin embargo, existen pocas evidencias que confirmen dicha relación. Las especies de rumiantes silvestres tienen cambios estacionales muy marcados en su función reproductiva y fuertes cambios anuales en sus concentraciones plasmáticas de testosterona. El presente trabajo ha examinado la influencia de cambios inducidos en las secreciones de testosterona sobre las variables espermáticas tras la congelación convencional y ultra-rápida, usando la cabra montés como modelo experimental. En el primer experimento, los niveles de testosterona fueron reducidos en medio de la estación reproductiva (diciembre) usando el antiandrógeno acetato de ciproterona (CA). En el segundo experimento, los niveles de testosterona han sido incrementados al final de la estación reproductiva (enero) mediante la administración del andrógeno propionato de testosterona (TP). Durante diciembre, las concentraciones de testosterona fueron superiores en el plasma sanguíneo y seminal de los animales no tratados que en los tratados con CA ($P<0,001$ y $P<0,05$; respectivamente). En comparación con los controles, los animales tratados con TP mostraban concentraciones de testosterona en sangre más altas, aunque las concentraciones de testosterona en plasma seminal eran más bajas durante enero ($P<0,01$ y $P<0,001$; respectivamente). El tamaño de las vesículas seminales de los machos tratados con TP era superior que en los animales no tratados ($P<0,05$). Cuando se administraba CA, la viabilidad espermática se incrementaba en comparación con el grupo control ($P<0,05$), independientemente del protocolo de congelación aplicado. Para el proceso de congelación ultra-rápida, el ratio de criorresistencia disminuyó para la motilidad cuando se administraba el TP ($P<0,05$). Los valores de las variables morfométricas para muestras en fresco disminuyeron durante los 50 días después del final del tratamiento con CA ($P<0.001$), y se incrementaron después del final de tratamiento con TP ($P<0,001$). Las variaciones en la concentración de testosterona parecen tener una influencia negativa sobre la criorresistencia espermática. Esto podría explicar los cambios estacionales observados en la congelación espermática en algunas especies, independientemente de la calidad espermática en fresco.

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ORIGINAL ARTICLE

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# Influence of circulating testosterone concentration on sperm cryoresistance: The ibex as an experimental model

Paula Bóveda[1] | Milagros Cristina Esteso[1] | Rosario Velázquez[1] | Cristina Castaño[1] | Adolfo Toledano-Díaz[1] | Antonio López-Sebastián[1] | Octavio Mejía[2] | María Gemma Millán de la Blanca[1] | Rodolfo Ungerfeld[3] | Julián Santiago-Moreno[1]

[1]Department of Animal Reproduction, INIA, Madrid, Spain

[2]Centro de Enseñanza, Investigación y Extensión en Producción Ovina, FMVZ-UNAM, Tres Marías, Morelos, Mexico

[3]Departamento de Biociencias Veterinarias, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay

**Correspondence**
Julián Santiago-Moreno, Department of Animal Reproduction, INIA, Avda. Puerta de Hierro km 5.9, 28040 Madrid, Spain.
Email: moreno@inia.es

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## Abstract

**Background:** Recent studies have noted that the circulating testosterone concentration may affect the ability of spermatozoa to survive cryopreservation. However, few attempts to confirm such a relationship have been made. Wild ruminant species have very marked seasonal changes in their reproductive function and strong annual changes in their plasma testosterone concentration.

**Objectives:** The present work examines the influence of induced changes in testosterone secretion on sperm variables following conventional slow freezing and ultra-rapid freezing, using the Iberian ibex as an experimental model.

**Materials and Methods:** In a first experiment, testosterone levels were reduced in the middle of the rutting season (December) using the antiandrogen cyproterone acetate (CA). In a second experiment, testosterone levels were increased at the end of the rutting season (January) via the use of the androgen testosterone propionate (TP).

**Results:** During December, the testosterone concentration was found to be higher in the blood and seminal plasma of untreated males than in those of CA-treated males ($p < 0.001$ and $p < 0.05$, respectively). Compared with controls, the TP-treated animals had higher blood plasma testosterone concentrations but lower seminal plasma testosterone concentrations during January ($p < 0.01$ and $p < 0.001$, respectively). The seminal vesicles of the TP-treated males were larger than those of untreated males ($p < 0.05$). When CA was administered, sperm viability improved compared with controls ($p < 0.05$), irrespective of the freezing protocol followed. For the ultra-rapid freezing procedure, the cryoresistance ratio for motility decreased when TP was administered ($p < 0.05$). The values for fresh sperm morphometric variables decreased during the 50 days after the end of CA treatment ($p < 0.001$) and increased over the same time after the end of TP treatment ($p < 0.001$).

**Discussion and Conclusion:** The circulating testosterone concentration appears to influence sperm cryoresistance. This may explain the seasonal changes seen in sperm freezability in some species, independent of fresh sperm quality.

KEYWORDS
accessory glands, cyproterone, cryopreservation, sperm freezability, testosterone

## 1 | INTRODUCTION

Sperm cryopreservation is routinely used to avoid subjecting azoospermic patients to repeated semen collection procedures,[1] to preserve spermatozoa before radio- and chemotherapy,[2] and as a back-up fertility measure for individuals who choose to undergo vasectomy.[3] Spermatozoon also needs to be cryopreserved to provide animal models for the study of human disease,[4] to maintain the genetic resources of rare domestic animals,[5] for use in animal breeding programs,[4] to improve traits that could help ensure global food security, and to establish germplasm banks for the conservation of threatened wild species.[6] The damage caused to spermatozoa by freezing-thawing varies according to the protocol followed (ie, the sperm selection technique, the extender, cooling rate, packaging, and thawing procedure used),[7,8] the source of the spermatozoa (eg, ejaculated, epididymal),[9] the semen collection method,[10] and the quality of the freshly collected samples. Initial sperm quality may vary according to the presence of underlying disease,[11] individual factors,[12] the season of semen collection (especially in species with seasonal breeding[13,14]), and the breed of certain domestic species.[15] In addition, the concept of "good and bad freezers" postulated by Watson might extend beyond variations in the semen of different individuals[16] to the endocrine status of the individual at the moment of semen collection, which may affect the ability of spermatozoa to survive cryopreservation.[17]

In most small ruminant species, the circulating testosterone concentration varies seasonally, affecting spermatogenesis and the functionality of sperm cells[18] and accessory glands.[19] High levels of testosterone during the rutting season appear to exert a harmful effect on the cryoresistance of ibex (*Capra pyrenaica*),[20] mouflon (*Ovis musimon*), and ram (*Ovis aries*)[17] spermatozoa. This suggests that the peak of the rutting season is not the best time to collect spermatozoa for cryopreservation.[14]

The influence of testosterone fluctuations on sperm freezability is difficult to evaluate, especially in species that are non-seasonal or moderately seasonal, for example, domesticated species that show a more continuous endocrine pattern as a result of human selection.[21] Other changes in physiological status over the year make it even harder to definitively associate any differences in sperm cryoresistance with changes in testosterone, and few studies attempting to demonstrate such a cause-effect relationship have been undertaken. However, manipulating the testosterone concentration artificially in individuals from which semen is collected allows the influence of the hormone to be isolated.

The treatment with antiandrogens affects all androgen-dependent organs and functions, such as accessory sexual glands, spermatogenesis, libido, and male secondary sexual characteristics.[22,23] The effects of the antiandrogen cyproterone acetate (CA), a pharmaco-hormonal compound with antiandrogenic, antigonadotropic, and progestational properties, on reproductive functions have been widely reported in humans.[24,25] For instance, the antiandrogenic properties of CA have been used in urological clinic for treatment of advanced human prostate cancer[26] or suppression of sexual disorders.[24] CA induces a marked reduction in sperm concentration and motility, plus a moderate reduction in the volume of seminal fluid and testicular germ cell numbers.[27,28] On the other hand, the administration of androgens (eg, testosterone propionate, TP) raises testosterone plasma concentrations but decreases the intratesticular testosterone concentrations, which are required for the maintenance of spermatogenesis.[29] In rams, the administration of TP improves many sperm kinetic variables in fresh samples.[18] In any case, the overall effect of both CA and TP may vary according to the concentration administered.

Wild ruminant species could prove to be an excellent model for such experiments as, unlike domestic species, they have gone through no domestication process that might have affected the neuroendocrine control of reproductive function.[30] In general, wild ruminants show marked reproductive seasonality and notable annual changes in their blood plasma testosterone concentration.[31]

The aim of the present work was to examine how changes in the testosterone concentration at key moments of the year influence sperm cryopreservation, using the ibex, a wild ruminant species, as a model. Testosterone levels were modified experimentally at the middle and end of the rutting season by administrating antiandrogens or androgens. As sensitivity to testosterone concentration-associated sperm damage could differ according to the cooling rate employed (sperm quality is usually more strongly affected by a very fast cooling rate),[9] sperm samples were cryopreserved following a conventional slow freezing and an ultra-rapid freezing protocol.

## 2 | MATERIAL AND METHODS

### 2.1 | Animal handling

The animals used in this work were 12 male Iberian ibexes aged 3–9 years. All were handled according to procedures approved by the INIA Ethics Committee (*Órgano Regulador de los Comités de Ética de Experimentación Animal*), and in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments. The same ethics committee also approved the present work (ref. ORCEEA 2014/027; Madrid Regional Government ref. PROEX 271/14). All the above animals were born and raised in captivity at the INIA Department of Animal Reproduction (Madrid, Spain). They were housed in a 250 $m^2$ enclosure with partial roof cover and fed Visan K-59 (Visan Ind. Zoot. S.A, Madrid, Spain), which contains 15% crude protein, 15.7% crude fiber, 4% crude fat, 10.6% crude ash, and 0.5% Na. This commercial feed was supplemented with barley grain, barley straw, and dry alfalfa. Water and vitamin/mineral blocks were available *ad libitum*. To minimize stress during experimental procedures, the animals were gradually accustomed to handling in the restraining stall (2 $m^2$) where they would later be anesthetized. Anesthesia was induced with intravenous detomidine (50 μg/kg Domosedan, Pfizer Inc., Amboise Cedex,

France), ketamine hydrochloride (0.5 mg/kg Imalgene-1000, Rhône Mérieux, Lyon, France), and tiletamine-zolazepam (0.5 mg/kg Zoletil-100, Virbac España S.A., Barcelona, Spain) and maintained with 1.5% isoflurane (Isobavet, Intervet/Schering-Plough Animal Health, Madrid, Spain) with oxygen supplementation via an endotracheal tube (flow rate 2.5 L/min). During anesthesia, all animals were monitored via pulse oximetry and capnography. Anesthesia was reversed after sperm collection using yohimbine hydrochloride (0.7 mg/kg: half intravenous and half intramuscular; Sigma Chemical Co. St. Louis, USA).

## 2.2 | Experimental procedures

Marked monthly changes in testosterone secretion are observed over the year in ibexes, which are characterized by baseline levels from January to August, start to rise in September, reaching their highest values in October and November, and begin to decrease in December (coinciding with the highest number of matings in the wild), reaching again basal levels in January.[14] Testosterone secretion was modified in the periods in which testosterone secretion is still high (Experiment 1) and when basal levels are attained (Experiment 2). Animals were grouped into three groups (two treated groups and one control group) allowing that the age variability among groups was similar. Control animals were the same in both Experiment 1 and Experiment 2.

### 2.2.1 | Experiment 1: Influence of the antiandrogen cyproterone acetate at mid-rutting season on sperm cryoresistance

This experiment was performed with eight animals and lasted from the end of November until the end of February. The animals were divided into two groups: (1) cyproterone acetate (CA) group with four animals that received 200 mg of CA (Androcur, Schering A.G., Berlin, Germany) intramuscularly diluted in 2 mL of olive oil (vehicle) twice weekly (Tuesday and Thursday) throughout December (coinciding with the period in which testosterone plasma concentrations were expected to be high),[32] and (2) control group composed of the other four ibexes. These received 2 mL olive oil intramuscularly without CA twice weekly on the same days as above. The CA dose was chosen according to Santiago-Moreno et al.[33] This protocol has been successfully used at our laboratory to maintain plasma testosterone concentrations at basal levels.

A total of seven semen collections were performed from each animal, with a 15-day interval between collections. The first sample was collected just before the start of treatment (November; used only for morphometric analysis), two were collected during treatment (December), and four after treatment had ended (January and February). Coinciding with semen collection, the diameter of the testes, and the area of the seminal vesicles and bulbourethral glands were measured by ultrasonography. Blood samples were collected once weekly throughout the experimental period to measure testosterone concentrations.

### 2.2.2 | Experiment 2: Influence of testosterone propionate at the end of the rutting season on sperm cryoresistance

This experiment was performed with eight animals and lasted from early January to the end of March. These animals were divided into two groups: (1) testosterone propionate (TP) group with four ibexes that received 25 mg of TP subcutaneously (Fluka, Sigma-Aldrich) diluted in 2 mL of olive oil (vehicle) twice weekly (Tuesday and Thursday) in January (coinciding with the seasonal fall in plasma testosterone), and (2) control group composed of the same control animals as in Experiment 1, which received 2 mL of olive oil without TP subcutaneously twice weekly (Tuesday and Thursday). The TP dose was chosen according to Santiago-Moreno et al.[33] This protocol has been successfully used at our laboratory to induce high plasma testosterone concentrations.

Like Experiment 1, semen was collected seven times from each animal, with a 15-day interval between collections. The first sample was collected just before beginning TP treatment (used only for morphometric analysis), two during treatment (January), and four after treatment had ended (February and March). Coinciding with semen collection, the testes, seminal vesicles, and bulbourethral glands were again measured by ultrasonography. Blood samples were collected once weekly throughout the experimental period to measure the testosterone concentration.

Data were grouped into those covering the period coinciding with CA or TP treatment, and the 50 days after the end of treatment—the time needed for spermatogenesis to complete in caprines.[34]

## 2.3 | Collection of blood samples and ultrasound analysis

Blood samples were collected by jugular venipuncture in heparinized tubes. Samples were centrifuged at 1500 *g* for 15 min, and the separated plasma was stored at −20°C until testosterone analysis.

Testicular diameter, the area of the bulbourethral glands and seminal vesicles, and the presence of spermatozoa in the ampulla of the vas deferens were examined by ultrasound using real-time transrectal ultrasonography employing a 7.5-MHz linear array probe (Prosound 2, Aloka Co., Ltd., Tokyo, Japan).[19]

## 2.4 | Sperm evaluation

Sperm samples were collected by transrectal ultrasound-guided massage of the accessory glands (TUMASG).[35,36] The diluents and

all other materials that came into contact with the semen were maintained at 37°C. The volume of the ejaculates was measured using a micropipette (Gilson, Villiers Le Bel, France). Sperm concentration was calculated before freezing using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany). Sperm motility was evaluated using a computer-aided sperm analysis system (CASA) (SCA, Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase-contrast microscope with negative contrast capability. The percentages of immotile spermatozoa, sperm showing progressive motility, and spermatozoa showing non-progressive motility were determined in a minimum of three fields and 500 sperm tracks (image acquisition rate 25 frames/s).[35] Morphological abnormalities were assessed by phase-contrast microscopic examination of glutaraldehyde-fixed samples. Sperm viability and acrosome status were assessed by fluorescence microscopy using a fluorochrome combination of propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC), as previously described.[37] These variables required the observation of 200 cells.

Finally, smears were prepared for morphometric analysis by placing 5 µl of the fresh semen on the clear end of a frosted slide and dragging the drop across it. The smears were allowed to air-dry before staining with Hemacolor and assessed by CASA using the morphometry module of Sperm-Class Analyzer v.5.3.0.1 software (Microptic S.L. Barcelona, Spain) as described by Esteso et al.[38] The percentage of the head occupied by the acrosome was measured together with the length, width, perimeter, and area of the sperm head. For this analysis, a total of 100 sperm heads were measured on each slide. All sperm variables, except morphometry, were assessed before and after freezing with both cryopreservation methods. Morphometric variables were measured in fresh semen in the samples collected before the treatments and during the 50 days after the end of treatment.

## 2.5 | Sperm cryopreservation

All semen samples were separated into two fractions and diluted 1:1 (vol:vol) with TCG (Tris 313.7 mm, citric acid 104.7 mm, glucose 30.3 mm) for centrifugation at 900 *g* for 20 min. After centrifugation, the supernatant was separated into another centrifuge tube (Sterilin, Stone, UK) and centrifuged a second time to separate out the seminal plasma. This was stored at −20°C until testosterone determination. In preparation for conventional slow freezing, sperm pellets were resuspended at room temperature (23°C) with TCG (Tris 313.7 mm, citric acid 104.7 mm, glucose 30.3 mm, 6% egg yolk [vol/vol], and 5% glycerol [vol/vol]) to a final concentration of $100 \times 10^6$ spermatozoa/mL. In preparation for ultra-rapid freezing, sperm pellets were similarly resuspended in the same extender but with glycerol substituted by 100 mM sucrose. All suspensions were adjusted to pH 6.8 with NaOH at room temperature. The osmolality (measured in the absence of cryoprotectants) of all suspensions was 345 mOsm/kg. All reagents used in the preparation of the extenders were purchased from Panreac Química S.A. (Barcelona, Spain) or the Sigma Chemical Co. (St. Louis, USA).

Sperm samples cryopreserved by conventional slow freezing were cooled at 5°C for 1 h, and then maintained at this temperature for 2 h. Aliquots were then loaded into 0.25-mL straws and frozen by placing them in nitrogen vapor 5 cm above the surface of a liquid nitrogen bath for 10 min.[39] The sperm samples cryopreserved by ultra-rapid freezing were cooled for 30 min at 5°C and then plunged drop by drop (about 50 µL/drop) directly into liquid nitrogen as reported by Pradiee et al.[40] All cryopreserved samples were kept in liquid nitrogen for 12 months until thawing. For the straws, this was performed using a water bath at 37°C for 30 s; for the pellets, it was achieved by placing them on a DPP70 thermo-regulated conical hotplate (INIA, Madrid, Spain) set at 60–65°C.

## 2.6 | Testosterone analysis

Testosterone concentrations in blood plasma and seminal plasma aliquots (250 µL and 50 µL respectively) were measured (in duplicate) by radioimmunoassay as previously described.[19] The sensitivity of the technique was 0.06 ng/mL; the intra-assay and inter-assay coefficients of variation were 7% and 11%, respectively.

## 2.7 | Statistical analysis

Data were expressed as means ±SE. The plasma testosterone data showed a skewed distribution as determined by the Shapiro-Wilk test; values were therefore log-transformed before analysis. The same log transformation was made for the ultrasound measurements of testicular diameter, and for the area of the bulbourethral glands and the seminal vesicles. The values for sperm variables expressed in percentages that showed non-normal distributions, as determined by the Shapiro-Wilk test, were arcsine-transformed before analysis. The remaining sperm variables showing a non-normal distribution were log-transformed or arcsinh-transformed (in case of variables including zero values). The cryoresistance ratio (CR) for different variables was determined as the *(value after thawing/value before thawing) x 100])*.[41]

The effects of CA (Experiment 1) and TP treatment (Experiment 2) on the plasma testosterone concentration, the area of the sexual accessory glands, and the CR values for the different sperm variables returned by each freezing method were assessed by GLM ANOVA. The effects of the CA (Experiment 1) and TP treatment (Experiment 2), and of their interaction with the data period (ie, data collected during and after treatment), on fresh sperm variables and the CR values returned by each freezing method, were studied by GLM repeated-measures ANOVA. Morphometric variables were analyzed by factorial ANOVA, examining the interaction between the treatment and sample collection period. Significance was set at $p < 0.05$. All calculations were performed

using Statistica for Windows v.12.0 software (StatSoft, Tulsa, OK, USA).

## 3 | RESULTS

### 3.1 | Experiment 1: Influence of CA treatment during the rutting season on sperm cryoresistance

During December, the testosterone concentration was higher in the blood and seminal plasma of the untreated than the CA-treated animals ($p < 0.001$ and $p < 0.05$, respectively) (Figure 1). In addition, the seminal vesicles of the CA-treated animals were smaller than those of the controls during December ($p < 0.001$) (Figure 2). No differences were seen between the CA and control animals in terms of testicular diameter or the area of the bulbourethral gland.

During the post-treatment period, the semen of the CA-treated males showed a greater percentage of spermatozoa with morphological abnormalities than did that of the untreated males ($p < 0.05$) (Table 1). During the period of treatment, the CR viability value was greater for the CA-treated than for the control animals irrespective of the freezing method used ($p < 0.05$; Table 2); no differences were seen in the post-treatment period. Moreover, during the treatment period, the CR motility value was greater for the CA-treated animals than for the controls ($p = 0.09$) when the spermatozoon was slow-frozen. During the post-treatment period, the CR-VCL value was

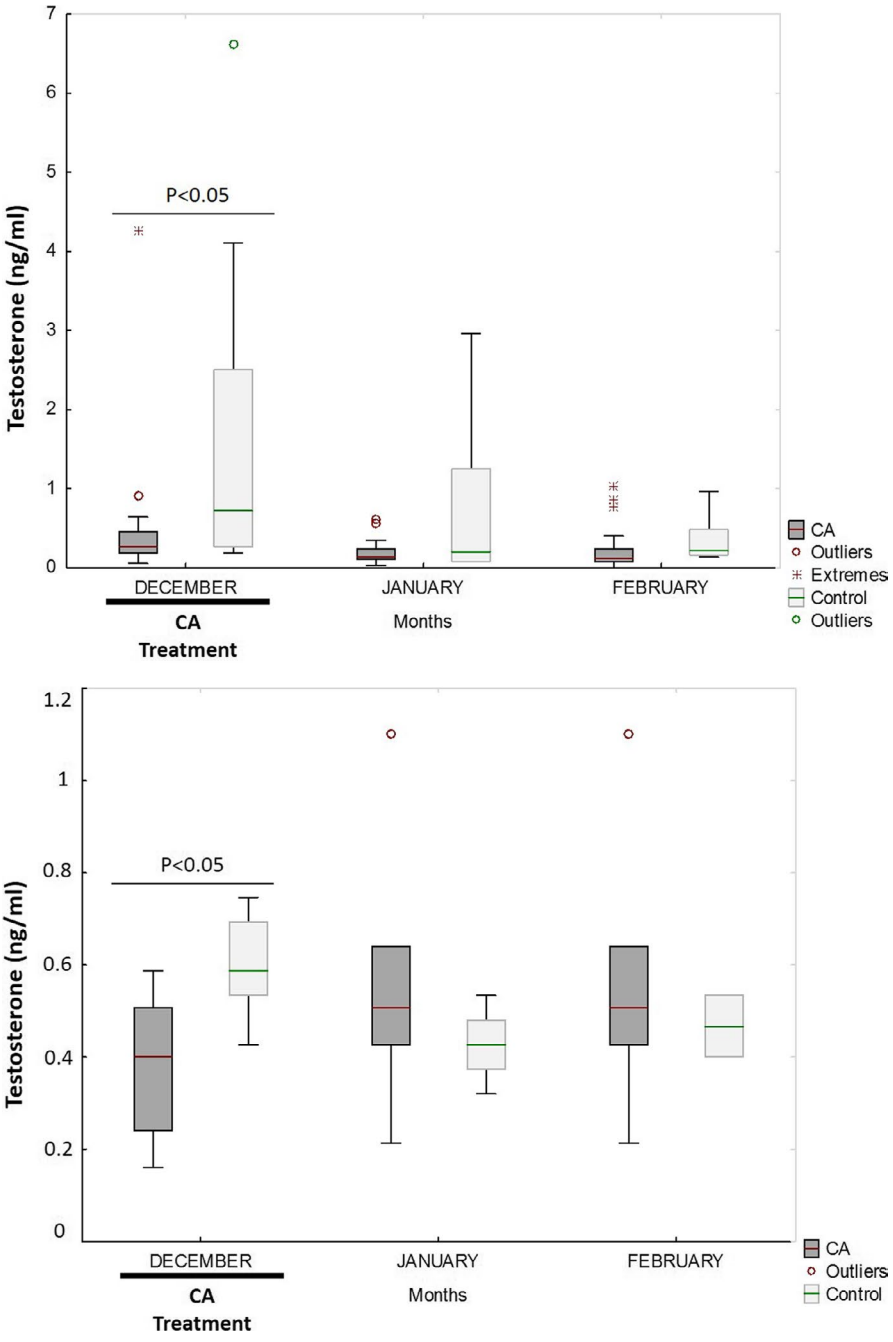


**FIGURE 1** Changes in blood plasma testosterone (top) and seminal plasma testosterone (bottom) from the beginning of cyproterone acetate (CA) treatment to two months post-treatment. The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line), and whiskers extend from the smallest up to the largest value

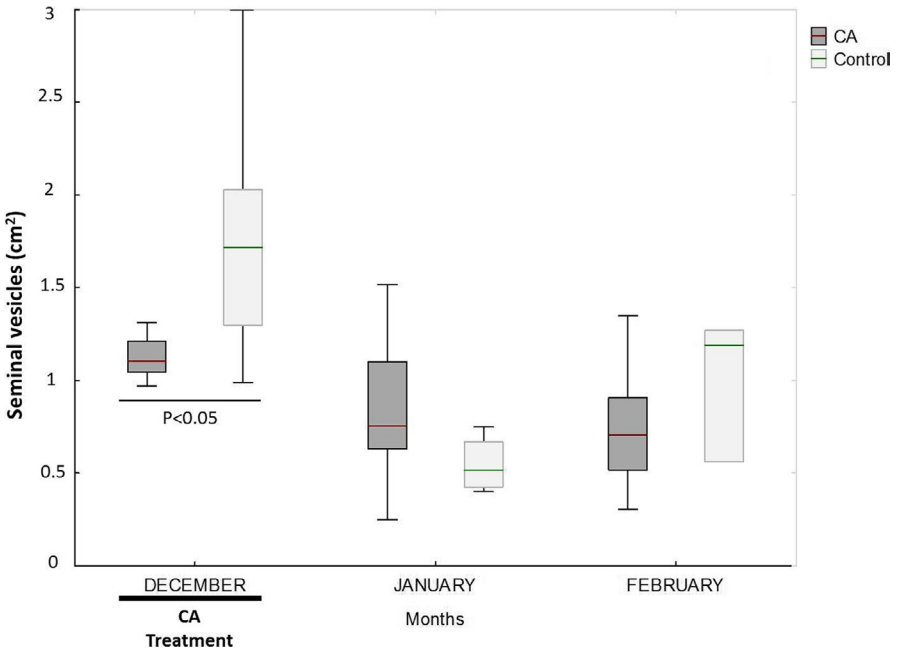


**FIGURE 2** Changes in seminal vesicle area, from the beginning of cyproterone acetate (CA) treatment to two months post-treatment. The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line), and whiskers extend from the smallest up to the largest value

**TABLE 1** Values (means ±SE) for fresh sperm variables in control and CA-treated animals

| | Fresh during treatment | | Fresh after treatment | |
|---|---|---|---|---|
| **Sperm variables** | **Control** | **CA** | **Control** | **CA** |
| Motility (%) | 59.4 ± 9.8 | 46.7 ± 7.8 | 38.3 ± 6.5 | 43.7 ± 11.4 |
| VCL (μm/s) | 79.5 ± 14.5 | 86.3 ± 9.4 | 80 ± 11.9 | 66 ± 10.4 |
| VSL (μm/s) | 39.3 ± 7.7 | 33.4 ± 4.9 | 35.2 ± 6.9 | 27.8 ± 6.3 |
| VAP (μm/s) | 55.7 ± 10.3 | 52 ± 7.1 | 51.4 ± 8.2 | 42 ± 9 |
| ALH (μm/s) | 2.7 ± 0.7 | 3.1 ± 0.5 | 3.3 ± 0.6 | 2.5 ± 0.6 |
| Viability (%) | 71.6 ± 11 | 67.4 ± 6.6 | 76.2 ± 4.8 | 76.8 ± 4.1 |
| Acrosome integrity (%) | 95 ± 2.1 | 87.9 ± 5.4 | 91 ± 3.1 | 92.2 ± 0.9 |
| Morphological abnormalities (%) | 45 ± 12.5 | 37.1 ± 6.8 | 33.5 ± 7.6[b] | 59.2 ± 6.4[a] |

Different letters indicate significant differences ($p < 0.05$) between the control group and the treatment group.

Abbreviations: ALH, amplitude of lateral head displacement; CA, cyproterone acetate; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

lower for the CA-treated animals than the controls when the spermatozoon was slow-frozen ($p < 0.01$) (Table 2). The CR-VSL and CR-VAP values also tended to be lower than in the controls ($p = 0.07$ for both), while the CR acrosome integrity tended to be higher ($p = 0.09$) (Table 2).

The fresh spermatozoa collected from the CA-treated animals had smaller heads than did those of the controls ($p < 0.001$) (Table 3).

### 3.2 | Experiment 2: Influence of TP treatment at the end of the rutting season on sperm cryoresistance

The TP-treated animals had higher blood plasma testosterone concentrations, but lower seminal plasma testosterone concentrations, than the control animals during January ($p < 0.01$ and $p < 0.001$, respectively) (Figure 3). No differences were seen in the following months. While the seminal vesicles of the TP-treated males were larger than those of the untreated males during January ($p < 0.05$) (Figure 4), no differences in testicular diameter or the area of the bulbourethral gland were seen at any time.

The fresh semen samples were not affected by the administration of TP during the treatment period. In the post-treatment period, the VSL and VAP were lower in the fresh spermatozoa of the treated males ($p < 0.05$) (Table 4). During the treatment period, the CR motility value was lower for the TP-treated than for the control males when the spermatozoon was ultra-rapidly frozen ($p < 0.05$) (Table 5).

The fresh sperm cells of the TP-treated animals were longer and had a greater head area and perimeter than did those of the control animals, both before treatment and in the post-treatment period ($p < 0.001$) (Table 6).

**TABLE 2** Cryoresistance ratios (means ±SE) for sperm variables in control and CA-treated animals following the different protocols

| | CR slow freezing | | | | CR ultra-rapid freezing | | | |
|---|---|---|---|---|---|---|---|---|
| | Frozen-thawed during treatment | | Frozen-thawed after treatment | | Frozen-thawed during treatment | | Frozen-thawed after treatment | |
| Sperm variables | Control | CA | Control | CA | Control | CA | Control | CA |
| Motility | 28.5 ± 6.1 | 54.1 ± 10.5 | 61.4 ± 13.7 | 60 ± 10.4 | 7.2 ± 0.8 | 6.7 ± 1.6 | 18.6 ± 4.4 | 13.2 ± 3 |
| VCL | 87.8 ± 17.2 | 69.3 ± 7.6 | 95.9 ± 6.2$^{a}$ | 65.5 ± 3.5$^{b}$ | 45.2 ± 4.9 | 59.8 ± 11.4 | 67.1 ± 11.4 | 53.7 ± 9.3 |
| VSL | 117.1 ± 26.6 | 109.6 ± 24.4 | 145.3 ± 26 | 83.8 ± 13 | 43.9 ± 12.8 | 50.4 ± 7.6 | 97.5 ± 25.5 | 55.2 ± 14.1 |
| VAP | 101.4 ± 22.4 | 87.1 ± 16.1 | 125.3 ± 22.4 | 72.1 ± 9.2 | 41.9 ± 9.5 | 56.2 ± 10.7 | 77.4 ± 17.7 | 48.9 ± 10.8 |
| ALH | 87.7 ± 14.4 | 73.5 ± 5.8 | 62.2 ± 7.3 | 77.4 ± 4.4 | 50.3 ± 18.6 | 42.6 ± 13 | 61.3 ± 13.1 | 46.6 ± 14.9 |
| Viability | 48.2 ± 10.8$^{b}$ | 91.4 ± 15$^{a}$ | 46.4 ± 7.2 | 46.4 ± 12.3 | 22.4 ± 8.7$^{B}$ | 44.7 ± 5.4$^{A}$ | 23.8 ± 3.5 | 33.4 ± 7.6 |
| Acrosome integrity | 60.7 ± 13 | 75.3 ± 5.9 | 66.3 ± 6.8 | 82.8 ± 3.1 | 52.5 ± 11.2 | 65 ± 6.9 | 69 ± 7.2 | 75.1 ± 5.5 |
| Normal spermatozoa | 112.4 ± 39.5 | 105.6 ± 8 | 63.8 ± 11.8 | 111.2 ± 35 | 97.1 ± 30.4 | 105.4 ± 7.1 | 66 ± 13 | 128.9 ± 35.5 |

Different letters indicate significant differences ($p < 0.05$) between the control group and the treatment group for slow freezing (lower case letters) and for ultra-rapid freezing (upper case letters).

Abbreviations: ALH, amplitude of lateral head displacement; CA, cyproterone acetate; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

**TABLE 3** Morphometric values (means ±SE) for fresh spermatozoa from control and CA-treated animals, before treatment and over 50 days post-treatment

| | Control before | CA before | Control 50 days post-treatment | CA 50 days post-treatment |
|---|---|---|---|---|
| Length (μm) | 8.25 ± 0.02 | 8.28 ± 0.02 | 8.07 ± 0.03 | 8.00 ± 0.02 |
| Width (μm) | 4.02 ± 0.01 | 4.02 ± 0.02 | 4.09 ± 0.02$^{A}$ | 4.02 ± 0.01$^{B}$ |
| Area (μm$^2$) | 27.52 ± 0.13 | 27.67 ± 0.11* | 27.41 ± 0.18$^{A}$ | 26.68 ± 0.12$^{B}$* |
| Perimeter (μm) | 21.23 ± 0.06 | 21.31 ± 0.04* | 21.12 ± 0.07$^{A}$ | 20.85 ± 0.05$^{B}$* |
| Acrosome (%) | 55.26 ± 0.33 | 55.89 ± 0.14 | 56.32 ± 0.18 | 56.52 ± 0.14 |

Different letters indicate significant differences ($p < 0.05$) between control and treatment groups post-treatment (upper case letters). Asterisks show significant differences ($p < 0.05$) between pre-treatment and 50 days post-treatment.

Abbreviations: CA, cyproterone acetate. Acrosome (%): percentage of the head occupied by the acrosome.

## 4 | DISCUSSION

The two treatments modified the plasma testosterone concentration as expected[33]—modifications that were associated with changes in sperm cryoresistance. The rapid reduction in blood plasma testosterone levels after CA treatment was similar to that observed in white-tailed deer,[42] fallow deer,[23] and domestic goat.[43] CA induced testosterone levels similar to those seen outside the reproductive season.[14] TP administration induced physiological high plasma testosterone concentrations as previously described in ibexes.[33] Plasma testosterone concentrations were highly variable with both CA and TP treatments. The reduced number of animals per group could have an influence on this variability. In addition, marked fluctuations, over relatively short periods, in peripheral plasma levels of testosterone secretion resulting from their intermittent secretion[44] should also be taken into account. A regimen of sampling more frequent should be used to avoid this problem.[19]

A reduction in the plasma testosterone concentration improved cryoresistance, while an increase worsened certain cryoresistance variables. For example, the CR viability value improved when plasma testosterone was reduced by the CA treatment, whereas CR motility fell after TP treatment. It is interesting that most of the differences in the CR values were observed during the time that the treatments were being administered; they were not generally maintained after the end of treatment. Unlike that previously proposed,[17] this suggests that they were not the consequence of modifying the early stages of spermatogenesis (which lasts about 48 days in goats).[34] Rather, they would appear to be mainly related to changes occurring during the transit of spermatozoa in the epididymal cap and the corpus epididymis, during the final maturation in the cauda epididymis, and during the short period in which the sperm cells are exposed to seminal vesicle secretion during ejaculation. The changes might involve the remodeling of the lipid and protein components of the plasma membrane.[45] The absorptive and secretory activity of the epididymal epithelium is regulated by androgens.[46] Epididymosomes[47] and extracellular vesicles[48] contribute in the addition and replacement of sperm proteins during epididymal transit and ejaculation, respectively, inducing strong variations in seminal plasma composition.

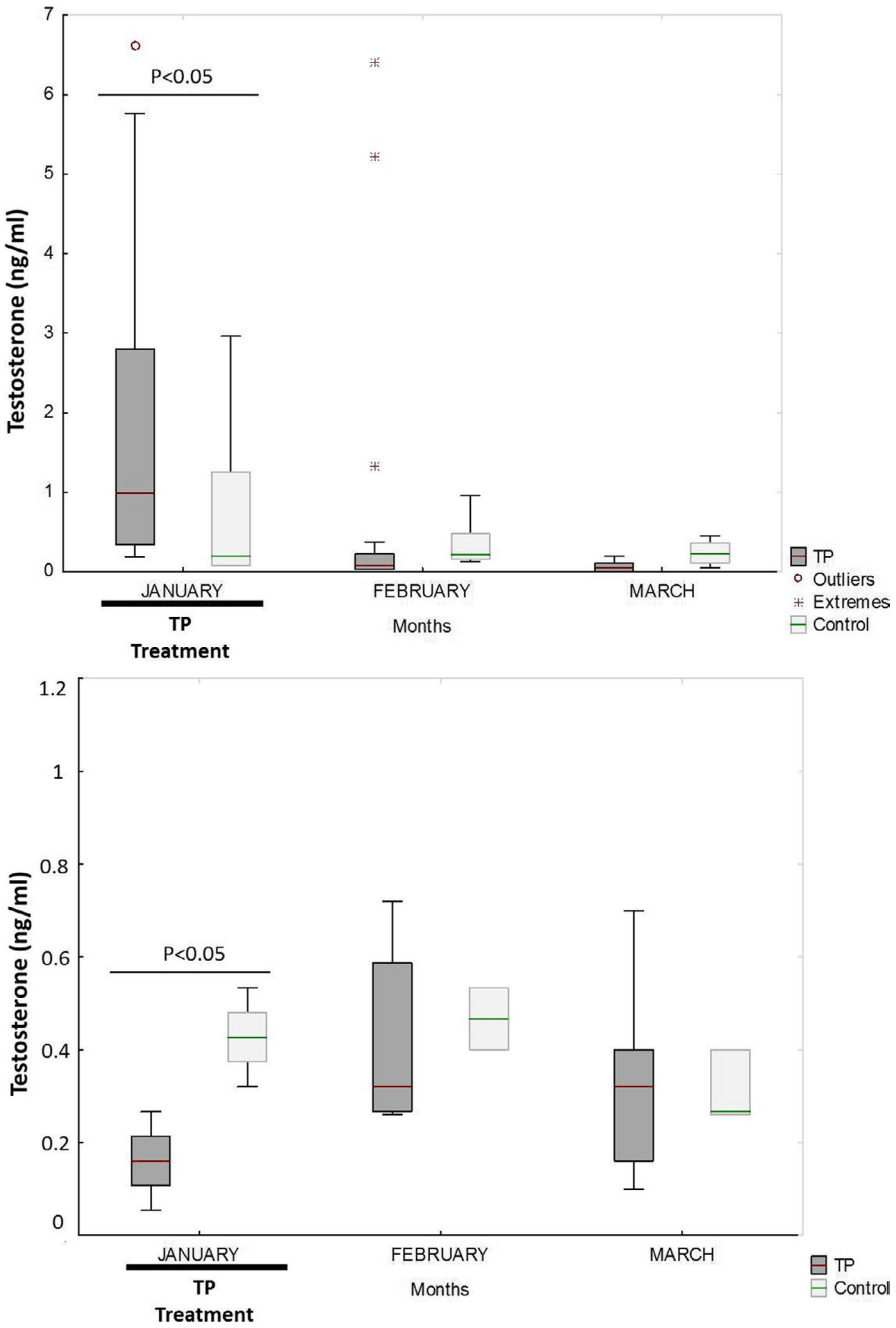


**FIGURE 3** Changes in blood plasma testosterone (top) and in seminal plasma testosterone (bottom), from the beginning of testosterone propionate (TP) treatment to two months post-treatment. The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line), and whiskers extend from the smallest up to the largest value

Certainly, testosterone can induce variations in the properties of sperm membranes,[49] and the negative effects on CR values of high concentrations might be due to changes in membrane fluidity and susceptibility to glycerol toxicity.[20] In rams, *in vitro* testosterone supplementation has been reported to reduce frozen-thawed acrosome integrity,[50] but in the present work, neither treatment modified the CR value for this variable.

Androgens have an important role in differentiation, development, and maintenance of epithelial cells in seminal vesicles; techniques of transmission electron microscopy and laser Doppler flowmetry have shown that glands' morphological features and blood flow, mainly in subepithelial capillaries, depend on androgens.[51] This may explain fact that CA treatment reduced the area of the seminal vesicles, while the TP treatment increased it.[52] Thus, besides their functional effects in the extra-testicular tract, the treatments appear to have had a direct impact on sperm CR values via their action at the level of the seminal vesicles. The reduction in the size of the seminal vesicles caused by CA treatment could have modified their activity[52] and therefore the composition of the seminal plasma. The possible reduction in glucose and fructose production by the seminal vesicles[53] during CA treatment might have influenced the metabolic status of the sperm cells, and perhaps their response to freezing-thawing. Further, in bulls the secretion of the seminal vesicles includes proteins, the synthesis of which is androgen-dependent. These bind to the sperm membrane[53] and might influence CR values.

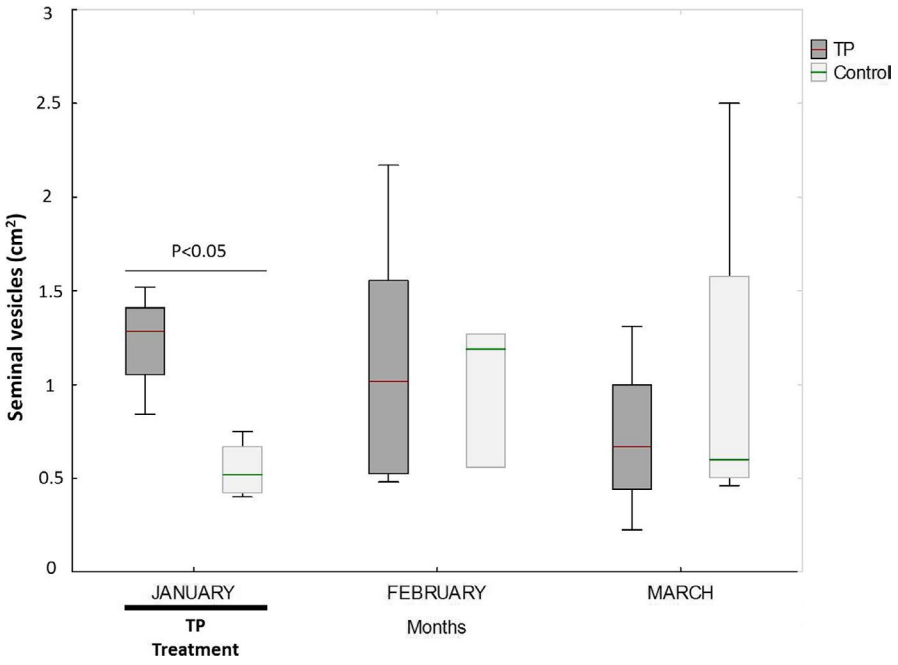


**FIGURE 4** Changes in seminal vesicle area, from the beginning of testosterone propionate (TP) treatment to two months post-treatment. The boxes spread form the 1st to the 3rd quartiles. Box plots show the median (horizontal line), and whiskers extend from the smallest up to the largest value

**TABLE 4** Values (means ±SE) for fresh sperm variables in control and TP-treated animals

| | Fresh during treatment | | Fresh after treatment | |
|---|---|---|---|---|
| **Sperm variables** | **Control** | **TP** | **Control** | **TP** |
| Motility (%) | 36.8 ± 4.1 | 50.4 ± 16.8 | 41.8 ± 13.1 | 31.8 ± 5.7 |
| VCL (μm/s) | 76.8 ± 42.8 | 71.3 ± 15 | 90 ± 12.6 | 66.3 ± 6.6 |
| VSL (μm/s) | 32 ± 21.5 | 32.6 ± 6 | 43.3 ± 8.3[a] | 24.8 ± 4.2[b] |
| VAP (μm/s) | 44.6 ± 23.9 | 50.4 ± 10.1 | 66.8 ± 11[a] | 40.8 ± 5.6[b] |
| ALH (μm/s) | 3.6 ± 1.9 | 2.8 ± 0.9 | 3 ± 0.3 | 2.4 ± 0.3 |
| Viability (%) | 78.8 ± 4 | 82.7 ± 11.8 | 68 ± 11.9 | 66.6 ± 6.4 |
| Acrosome integrity (%) | 90.5 ± 10.5 | 97.3 ± 2.1 | 93.3 ± 0.9 | 82.9 ± 3.2 |
| Morphological abnormalities (%) | 35.3 ± 24 | 42 ± 6.9 | 48.8 ± 12.6 | 51.5 ± 3.7 |

Different letters indicate significant differences ($p < 0.05$) between the control group and the treatment group.

Abbreviations: ALH, amplitude of lateral head displacement; TP, testosterone propionate; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

During the post-treatment period, the sperm heads were smaller in fresh samples collected from CA-treated individuals, suggesting that the spermatogenic cycle might have been affected. The sperm heads increased in size after the TP treatment, coinciding with that reported for domestic bucks.[18] These findings suggest that higher concentrations of testosterone lead to a larger head size. Certainly, a close relationship exists between blood plasma testosterone and the dimensions of human sperm heads.[54] During the non-breeding season of rams, that is, when the testosterone concentration is low, the size of the sperm head is also reduced,[55] probably due to changes in the activity of the germinal epithelium and Sertoli cells.[17] It is also possible that testosterone levels modify the organization of the manchette, a transient microtubular platform seen in elongating spermatids that plays an important role in head shaping.[56] The influence of head dimensions in susceptibility to freezing-thawing is matter of debate,[41] but small sperm cells have been reported less likely to be damaged by cryopreservation.[39,57] However, the present results show that, similar to that reported for some other wild species,[41] a smaller head size does not appear to be associated with variations in CR values. It remains to be determined how different factors may interact to modify sperm head size and its relationship with sperm CR values.

During the post-treatment period, the CR-VCL value was reduced in the spermatozoa of CA-treated males that were subjected to slow freezing. This might be explained in that this is the period during which the percentage of sperms with morphological abnormalities increases.[25] Certainly, the increase in morphological abnormalities observed in the present work during the post-treatment period would appear to reflect an effect of low testosterone levels on spermatogenesis. Surprisingly, and unlike that seen for blood plasma testosterone, the seminal plasma testosterone concentration was lower (compared to controls) in the TP-treated animals

**TABLE 5** Cryoresistance ratios (means ±SE) for sperm variables in control and TP-treated animals

| | CR slow freezing | | | | CR ultra-rapid freezing | | | |
|---|---|---|---|---|---|---|---|---|
| | Frozen-thawed during treatment | | Frozen-thawed after treatment | | Frozen-thawed during treatment | | Frozen-thawed after treatment | |
| Sperm variables | Control | TP | Control | TP | Control | TP | Control | TP |
| Motility | 62.9 ± 21.6 | 40.1 ± 7.3 | 41.8 ± 11 | 42.9 ± 6.1 | 18.3 ± 4.4$^{A}$ | 1.9 ± 0.4$^{B}$ | 17.9 ± 6.7 | 11.6 ± 1.5 |
| VCL | 96.9 ± 9.1 | 85.1 ± 22.8 | 65.7 ± 16.8 | 90 ± 12.2 | 67.6 ± 16.3 | 36.4 ± 12 | 47.4 ± 14 | 63.5 ± 7.5 |
| VSL | 159.8 ± 38.5 | 112.5 ± 38.8 | 76.7 ± 27.3 | 163.7 ± 40.8 | 101.9 ± 31.3 | 34.7 ± 17.1 | 59.9 ± 31.5 | 75.2 ± 13.8 |
| VAP | 135.2 ± 33.1 | 92.6 ± 28.6 | 68.7 ± 22.6 | 116.6 ± 25.4 | 85.4 ± 23 | 27.7 ± 11.4 | 43.2 ± 18.7 | 64.2 ± 10.9 |
| ALH | 59.2 ± 11.1 | 62.27 ± 31.2 | 43.2 ± 14.6 | 53 ± 10.9 | 55.1 ± 19.6 | 19.1 ± 14.2 | 42.7 ± 18.1 | 21.2 ± 6 |
| Viability | 49.9 ± 10.8 | 64.3 ± 8.4 | 47.9 ± 9 | 67.6 ± 13.1 | 27.2 ± 4.3 | 45.8 ± 22.1 | 18.9 ± 3.3 | 29.9 ± 3.5 |
| Acrosome integrity | 70.1 ± 9.8 | 60 ± 16.7 | 66.1 ± 5.8 | 80.8 ± 3.5 | 73.8 ± 9.4 | 49.9 ± 18.3 | 65.1 ± 8.5 | 72.3 ± 4 |
| Normal spermatozoa | 54.7 ± 14.8 | 98.8 ± 40.4 | 126.7 ± 44.6 | 81.5 ± 6.9 | 56.1 ± 15.8 | 94.5 ± 45.1 | 125.6 ± 48.6 | 82.5 ± 9 |

Different letters indicate significant differences ($p < 0.05$) between control and treatment groups for ultra-rapid freezing (upper case letters).
Abbreviations: TP, testosterone propionate; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement.

**TABLE 6** Morphometric values (means ±SE) for fresh spermatozoa from control and TP-treated animals, before treatment and over 50 days post-treatment

| | Control before | TP before | Control 50 days post-treatment | TP 50 days post-treatment |
|---|---|---|---|---|
| Length (μm) | 8.07 ± 0.03$^{b}$ | 8.37 ± 0.02$^{a*}$ | 8.05 ± 0.02$^{B}$ | 8.24 ± 0.02$^{A*}$ |
| Width (μm) | 4.09 ± 0.02 | 4.04 ± 0.01* | 4.16 ± 0.01 | 4.20 ± 0.02* |
| Area (μm$^2$) | 27.41 ± 0.18$^{b}$ | 27.99 ± 0.10$^{a*}$ | 27.83 ± 0.12$^{B}$ | 28.60 ± 0.15$^{A*}$ |
| Perimeter (μm) | 21.12 ± 0.07$^{b}$ | 21.60 ± 0.04$^{a}$ | 21.12 ± 0.05$^{B}$ | 21.50 ± 0.06$^{A}$ |
| Acrosome (%) | 56.32 ± 0.18 | 56.28 ± 0.14 | 56.10 ± 0.14 | 56.48 ± 0.13 |

Different letters indicate significant differences ($p < 0.05$) between control and treatment groups before treatment (lower case letters) and post-treatment (upper case letters). Asterisks show significant differences ($p < 0.05$) between pre-treatment and 50 days post-treatment.
Abbreviations: TP, testosterone propionate. Acrosome (%): percentage of the head occupied by the acrosome.

during the treatment period. This apparent lack of an association in the changes in blood and seminal plasma agrees with that reported for TP-treated domestic bucks.[18] Indeed, it has been previously reported that the seminal plasma testosterone concentration need not correlate with the blood plasma concentration.[58] It may be that testosterone is locally metabolized into other androgens, such as dihydrotestosterone and androstenedione,[59,60] with a more direct *in situ* action.

Although the model used in the present study (wild ruminants) does not allow the use of a large number of animals in the experiments, to increase the statistical power, it provided valuable information about the role of endocrine status on sperm response to freezing-thawing process. Future experiments should be designed to determine the mechanistic action of testosterone on sperm freezability. For instance, aquaporins (AQPs) adapt their membrane domain location to osmotic changes during freezing-thawing that can relate to variations in sperm cryosurvival.[61] The expression of some AQPs is regulated by androgens in certain cells, for example, uterine cells[62] or kidney cells,[63] and thus, a possible influence of testosterone on AQP expression in the sperm cell should not be ruled out.

In conclusion, testosterone appears to negatively influence sperm cryoresistance independent of sperm head size. This may explain why sperm freezability varies seasonally in some species, independent of its fresh quality. The present results provide insight into the use of testosterone as a biological marker for predicting how spermatozoa will react to freezing-thawing, which could facilitate the optimization of cryopreservation techniques by endocrine manipulation of testosterone levels.

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## CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

## AUTHORS' CONTRIBUTIONS

PB collected data, performed statistical analyses, and wrote the first draft of the manuscript. MCE and ASLS designed the study. RV, CC, ATD, OM, and MGMB collected data. RU interpreted results and revised the manuscript critically. JSM designed the study, performed statistical analyses, and wrote the manuscript. All authors and co-authors approved the final version of the manuscript to be published.

## ORCID

*Julián Santiago-Moreno* https://orcid.org/0000-0001-5551-8120

## REFERENCES

1. Tournaye H. Surgical sperm recovery for intracytoplasmic sperm injection: Which method is to be preferred? *Hum Reprod*. 1999;14(1):71-81.
2. Tournaye H, Goossens E, Verheyen G, et al. Preserving the reproductive potential of men and boys with cancer: current concepts and future prospects. *Hum Reprod Update*. 2004;10:525-532.
3. Silbert JA. Large-scale preservation of human semen. *Cryobiology*. 1972;9:556-558.
4. Groeneveld LF, Gregusson S, Guldbrandtsen B, et al. Domesticated animal biobanking: land of opportunity. *PLoS Biol*. 2016;14(7):e1002523.
5. Thélie A, Bailliard A, Seigneurin F, Zerjal T, Tixier-Boichard M, Blesbois E. Chicken semen cryopreservation and use for the restoration of rare genetic resources. *Poult Sci*. 2019;98:447-455.
6. Prieto MT, Sanchez-Calabuig MJ, Hildebrandt TB, Santiago-Moreno J, Saragusty J. Sperm cryopreservation in wild animals. *Eur J Wildl Res*. 2014;60:851-864.
7. Di Santo M, Tarozzi N, Nadalini M, Borini A. Human Sperm Cryopreservation: Update on Techniques, Effect on DNA Integrity, and Implications for ART. *Advan Urol*. 2012;2012:1-12.
8. Sharma R, Kattoor AJ, Ghulmiyyah J, Agarwal A. Effect of sperm storage and selection techniques on sperm parameters. *Syst Biol Reproduct Med*. 2015;61(1):1-12.
9. Martínez-Fresneda L, Castaño C, Bóveda P, et al. Epididymal and ejaculated sperm differ on their response to the cryopreservation and capacitation processes in mouflon (*Ovis musimon*). *Sci Rep*. 2019a;9:15659.
10. Jiménez-Rabadán P, Soler AJ, Ramón M, et al. Influence of semen collection method on sperm cryoresistance in small ruminants. *Anim Reprod Sci*. 2016;167:103-108.
11. Caponecchia L, Cimino G, Sacchetto R, et al. Do malignant diseases affect semen quality? Sperm parameters of men with cancers. *Andrologia*. 2016;48(3):333-340.
12. Mortimer D. *Practical laboratory andrology*. New York, NY: Oxford University Press; 1994.
13. Janett FR, Bettschen S, Burger D, Hassig M. Seasonal changes of semen quality and freezability in Franches-Montagnes stallions. *Anim Reprod Sci*. 2003;77(3–4):213-221.
14. Coloma MA, Toledano-Díaz A, Castaño C, et al. Seasonal variation in reproductive physiological status in the Iberian ibex (*Capra pyrenaica*) and its relationship with sperm freezability. *Theriogenology*. 2011;76:1695-1705.
15. Santiago-Moreno J, Bernal B, Pérez-Cerezales S, et al. Seminal plasma amino acid profile in different breeds of chicken: role of seminal plasma on sperm cryoresistance. *PLoS One*. 2019;14(1):e0209910.
16. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil*. 1995;7:871-891.
17. Martínez-Fresneda L, O'Brien E, Velázquez R, et al. Seasonal variation in sperm freezability associated with changes in testicular germinal epithelium in domestic (*Ovis aries*) and wild (*Ovis musimon*) sheep. *Reprod Fertil Dev*. 2019b;31:1545-1557.
18. Flores-Gil VN, Millan de la Blanca MG, Velázquez R, Toledano-Díaz A, Santiago-Moreno J, López-Sebastián A. Influence of testosterone administration at the end of the breeding season on sperm cryoresistance in rams (*Ovis aries*) and bucks (*Capra hircus*). *Domest Anim Endocrinol*. 2020;72:106425.
19. Santiago-Moreno J, Gómez-Brunet A, González-Bulnes A, Toledano-Díaz A, Malpaux B, López-Sebastián A. Differences in reproductive pattern between wild and domestic rams are not associated with inter-specific annual variations in plasma prolactin and melatonin concentrations. *Domest Anim Endocrinol*. 2005;28:416-429.
20. Coloma MA, Gómez-Brunet A, Velázquez R, Toledano-Díaz A, López-Sebastián A, Santiago-Moreno J. Freezability of Iberian ibex (*Capra pyrenaica*) sperm according to the glycerolization temperature and plasma testosterone concentration. *Cryobiology*. 2010;61:204-210.
21. Curlewis JD. Seasonal prolactin secretion and its role in seasonal reproduction: a review. *Reprod Fertil Dev*. 1992;4:1-23.
22. Neumann F, Töpert M. Pharmacology of antiandrogens. *Journal of steroid biochemistry*. 1986;25(5):885-895.
23. Kolle R, Kierdorf U, Fischer K. Effects of an antiandrogen treatment on morphological characters and physiological functions of male fallow deer (*Dama dama L.*). *J Exp Zool*. 1993;267(3):288-298.
24. Neumann F. The antiandrogen cyproterone acetate: discovery, chemistry, basic pharmacology, clinical use and tool in basic research. *Exp Clin Endocrinol*. 1994;102:1-32.
25. Fredricsson B, Carlström K. Effects of low doses of cyproterone acetate on sperm morphology and some other parameters of reproduction in normal men. *Andrologia*. 1981;13(4):369-375.
26. Guo C, Yeh S, Niu Y, et al. Targeting androgen receptor versus targeting androgens to suppress castration resistant prostate cancer. *Cancer Lett*. 2017;397:133-143.
27. Morse HC, Leach DR, Rowley MJ, Heller CG. Effect of cyproterone acetate on sperm concentration, seminal fluid volume, testicular cytology and levels of plasma and urinary ICSH, FSH and testosterone in normal men. *J Reprod Fertil*. 1973;32:365-378.
28. Koch UJ, Lorenz F, Danehl K, et al. Continuous oral low-dosage cyproterone acetate for fertility regulation in the male? – A trend analysis in 15 volunteers -. *Contraception*. 1976;14:117-135.
29. Morse HC, Horike N, Rowley MJ, Heller CG. Testosterone concentrations in testes of normal men: effects of testosterone propionate administration. *J Clin Endocrinol Metab*. 1973;37(6):882-886.
30. Gómez-Brunet A, Santiago-Moreno J, del Campo A, et al. Endogenous circannual cycles of ovarian activity and changes in prolactin and melatonin secretion in wild and domestic female sheep maintained under a long day photoperiod. *Biol Reprod*. 2008;78:552-562.
31. Lincoln G. Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*Ovis musimon*). *Anim Reprod Sci*. 1998;53:87-105.
32. Santiago-Moreno J, Gomez A, Toledano A, Gonzalez A, Lopez-Sebastian A. Seasonal endocrine changes and breeding activity in Mediterranean wild ruminants. *Reprod Dom Anim*. 2006;41(Suppl 2):72-81.
33. Santiago-Moreno J, Gómez-Brunet A, Toledano-Díaz A, Salas-Vega R, Gómez-Guillamón F, López-Sebastián A. Role of testosterone and photoperiod on seasonal changes in horn growth and sperm variables in the Iberian ibex, a model for polygynous wild bovids. *J Endocrinol*. 2012;214:155-163.
34. França LR, Becker-Silva SC, Chiarini-Garcia H. The length of the cycle of seminiferous epithelium in goats (*Capra hircus*). *Tissue Cell*. 1999;31:274-280.

35. Santiago-Moreno J, Castaño C, Toledano-Díaz A, et al. Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. *Theriogenology*. 2013;79:383-391.
36. Ungerfeld R, López-Sebastián A, Esteso MC, et al. Physiological responses and characteristics of sperm collected after electroejaculation or transrectal ultrasound-guided massage of the accessory sex glands in anesthetized mouflons (*Ovis musimon*) and Iberian ibexes (*Capra pyrenaica*). *Theriogenology*. 2015;84:1067-1074.
37. Soler AJ, Esteso MC, Fernández-Santos MR, Garde JJ. Characteristics of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa cryopreserved after storage at 5 °C in the epididymis for several days. *Theriogenology*. 2005;64:1503-1517.
38. Esteso MC, Rodríguez E, Toledano-Díaz A, et al. Descriptive analysis of sperm head morphometry in Iberian ibex (*Capra pyrenaica*): optimum sampling procedure and staining methods using Sperm-Class Analyzer®. *Anim Reprod Sci*. 2015;155:42-49.
39. Pradiee J, O'Brien E, Esteso MC, et al. Effect of shortening the pre-freezing equilibration time with glycerol on the quality of chamois (*Rupicapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates. *Anim Reprod Sci*. 2016;171:121-128.
40. Pradiee J, Esteso MC, López-Sebastián A, et al. Successful ultra-rapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa. *Theriogenology*. 2015;84:1513-1522.
41. O'Brien E, Esteso MC, Castaño C, et al. Effectiveness of ultra-rapid cryopreservation of sperm from endangered species, examined by morphometric means. *Theriogenology*. 2019;129:160-167.
42. Bubenik GA, Schams D, Coenen G. The effect of artificial photoperiodicity and antiandrogen treatment on the antler growth and plasma levels of LH, FSH, testosterone, prolactin and alkaline phosphatase in the male white-tailed deer. *Comp. Biochem. Physiol*. 1987;87:551-559.
43. Flores-Gil VN, Velázquez R, Toledano-Díaz A, Santiago-Moreno J, López-Sebastián A. Short term administration of cyproterone acetate for contraception: effects on testosterone secretion and semen characteristics in rams (*Ovis aries*) and bucks (*Capra hircus*). *Spanish Journal of Agricultural Research*. 2020;18(4):e0407.
44. Pelletier J, Garnier DH, de Revievers MM, Terqui M, Ortavant R. Seasonal variation in LH and testosterone release in rams of two breeds. *J Reprod Fertil*. 1982;64:341-346.
45. Jones R. Plasma membrane structure and remodeling during sperm maturation in the epididymis. *J. Reprod. Fertil*. 1998;53:73-84.
46. Brooks DE. Epididymal functions and their hormonal regulation. *Aust J Biol Sci*. 1983;36(3):205-222.
47. Nixon B, De Iuliis GN, Hart HM, et al. Proteomic profiling of mouse epididymosomes reveals their contributions to post-testicular sperm maturation. *Mol Cell Proteomics*. 2019;18:91-108.
48. Leahy T, Rickard JP, Pini T, Gadella BM, De Graaf SP. Quantitative proteomic analysis of seminal plasma, sperm membrane proteins and seminal extracellular vesicles suggests vesicular mechanisms aid in the removal and addition of proteins to the ram sperm membrane. *Proteomics*. 2020;20(12):1900289.
49. Purohit SB, Saxena D, Laloraya M, Kumar GP. Altered molecular dynamics and antioxidant status in the spermatozoa in testosterone-induced oligospermia in mouse. *Mol Reprod Dev*. 2000;55(3):316-325.
50. Martínez-Fresneda L, O'Brien E, López Sebastián A, et al. In vitro supplementation of testosterone or prolactin affects spermatozoa freezability in small ruminants. *Domest Anim Endocrinol*. 2019c;106372.
51. Ono Y, Suzuki K, Kashiwagi B, et al. Role of androgen on blood flow and capillary structure in rat seminal vesicles. *Tohoku J Exp Med*. 2004;202(3):193-201.
52. Prasad MR, Rajalakshmi M, Reddy PR. Action of cyproterone acetate on male reproductive functions. *Gynecol Invest*. 1971;2:202-212.
53. Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*, 2nd edn, vol. 18. NY, USA: Raven Press Ltd; 1994:1063-1175.
54. Garrett C, Liu DY, Mclachlan RI, Baker HWG. Time course of changes in sperm morphometry and semen variables during testosterone-induced suppression of human spermatogenesis. *Hum Reprod*. 2005;20(11):3091-3100.
55. Martí JI, Aparicio IM, Leal CLV, García-Herreros M. Seasonal dynamics of sperm morphometric subpopulations and its association with sperm quality parameters in ram ejaculates. *Theriogenology*. 2012;78:528-541.
56. Lehti MS, Sironen A. Formation and function of the manchette and flagellum during spermatogenesis. *Reproduction*. 2016;151:43-54.
57. Esteso MC. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples. *J Androl*. 2006;27:662-670.
58. Hampl R, Kubátová J, Sobotka V, Heráček J. Steroids in semen, their role in spermatogenesis, and the possible impact of endocrine disruptors. *Horm Mol Biol Clin Investig*. 2013;13(1):1-5.
59. Zalata A, Hafez T, Verdonck L, Vermeulen L, Comhaire F. Androgens in seminal plasma: markers of the surface epithelium of the male reproductive tract. *Int J Androl*. 1995;18:271-277.
60. Mann T, Lutwak-Mann C. Biochemistry of seminal plasma and male accessory fluids; application to andrological problems. In: Mann T, Lutwak-Mann C, eds. *Male Reproductive Function and Semen*. Berlin Heidelberg New York: Springer-Verlag; 1981:269-336.
61. Yeste M, Morató R, Rodríguez-Gil JE, Bonet S, Prieto-Martínez N. Aquaporins in the male reproductive tract and sperm: Functional implications and cryobiology. *Reprod Domest Anim*. 2017;52:12-27.
62. Salleh N, Mokhtar HM, Kassim NM, Giribabu N. Testosterone induces increase in aquaporin (AQP)-1, 5, and 7 expressions in the uteri of ovariectomized rats. *J Memb Biol*. 2015;248(6):1097-1105.
63. Loh SY, Giribabu N, Salleh N. Effects of gonadectomy and testosterone treatment on aquaporin expression in the kidney of normotensive and hypertensive rats. *Exp Biol Med*. 2017;242(13):1376-1386.


## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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# ARTÍCULO IV

## Influence of changes in prolactin secretion on sperm head dimensions and freezability of wild ruminants

*Andrologia (en revisión)*

## Influencia de los cambios de secreción de prolactina en las dimensiones de la cabeza y congelabilidad de los espermatozoides de los rumiantes silvestres

## RESUMEN ARTÍCULO IV

Este trabajo ha evaluado la influencia de los cambios en la secreción de prolactina sobre la criorresistencia espermática en cabra montés y muflón. Las concentraciones de prolactina fueron modificadas, en un primer experimento, mediante el uso de bromocriptina (BCR), un agonista dopaminérgico, fuera de la estación reproductiva; en un segundo experimento se modificaron mediante la administración de sulpirida, un antagonista dopaminérgico $D_2$, durante la estación reproductiva. Las muestras espermáticas fueron criopreservadas siguiendo protocolos de congelación convencional y congelación ultra-rápida. El tratamiento con BCR disminuía las concentraciones plasmáticas de PRL, mientras que el tratamiento con SLP las incrementaba. Los valores de criorresistencia para la velocidad curvilínea, velocidad rectilínea y velocidad media de trayectoria, fueron inferiores en los muflones tratados con BCR ($P<0,05$) que en los controles cuando los espermatozoides se congelaban de manera convencional, mientras que los valores de criorresistencia para la motilidad y para los espermatozoides normales eran mejores para los muflones tratados con BCR que los controles, cuando los espermatozoides se congelaban de manera ultra-rápida. El tratamiento con BCR determinaba un incremento de las dimensiones de la cabeza espermática en la cabra montés ($P<0,001$); en cambio, el tratamiento con BCR producía una disminución las dimensiones de la cabeza en los muflones ($P<0,001$). Los valores de criorresistencia para la motilidad, la amplitud lateral de la cabeza (ALH), viabilidad, e integridad de acrosoma, fueron inferiores en los muflones tratados con SLP que en los controles, cuando los espermatozoides se congelaban convencionalmente; los valores de criorresistencia para la viabilidad y la integridad del acrosoma fueron inferiores en comparación con los controles ($P<0,05$), cuando ese utilizaba la congelación ultra-rápida. En la cabra montés, la criorresistencia para la ALH era más baja para los machos tratados con SLP que los controles ($P<0,05$). El tratamiento con SLP inducía un incremento de las dimensiones de la cabeza espermática en la cabra montés ($P<0,001$), mientras que no afectaba al tamaño de cabeza espermática en los muflones. Los resultados sugieren que niveles elevados de PRL afectan negativamente a la criorresistencia de espermatozoides de la cabra montés y el muflón.

# Influence of changes in prolactin secretion on sperm head dimensions and freezability of wild ruminants

P. Bóveda[1], R. Velázquez[1], L. Martínez-Fresneda[1], O. Mejía[2], M. Oteo[3], A. Toledano-Díaz[1], C. Castaño[1], M.C. Esteso[1], R. Ungerfeld[4], A. López-Sebastián[1], J. Santiago-Moreno[1,*]

[1] Department of Animal Reproduction, INIA, Madrid, Spain

[2] Centro de Enseñanza, Investigación y Extensión en Producción Ovina, FMVZ-UNAM, Morelos, México

[3] Unidad de Aplicaciones Biomédicas y Farmacocinética. CIEMAT, Madrid, Spain

[4] Departamento de Fisiología, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay

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**Short title**

Prolactin and sperm cryoresistance

**Correspondence**

Julian Santiago-Moreno, Departamento de Reproducción Animal, INIA, Avda. Puerta de Hierro km 5.9, 28040 Madrid, Spain

Tel.: + 34 91 347420

Fax: + 34 91 3474014

E-mail: moreno@inia.es

**Summary**

This work examines the influence of induced changes in prolactin (PRL) secretion on sperm cryoresistance (CR) of ibex and the mouflon. PRL secretion was modified in a first experiment by the use of dopamine agonist bromocriptine (BCR) out the rutting season, and in a second experiment by the use

of dopamine $D_2$-receptor antagonist sulpiride (SLP) during the rutting season. Sperm samples were cryopreserved following a conventional slow freezing and an ultra-rapid freezing protocol. BCR treatment decreased plasma PRL concentrations, whereas SLP treatment increased it. CRs for curvilinear velocity, straight-line velocity, and average path velocity in BCR-treated mouflons were lower ($P<0.05$) than in controls when the sperm was slow-frozen, while CR-motility and CR for morphological normal sperm of BCR-treated mouflons were greater ($P<0.05$) than controls when the sperm was ultra-rapid frozen. BCR treatment increased the head sperm dimensions in ibexes ($P<0.001$); conversely, BCR treatment decreased the head dimensions in mouflons ($P<0.001$). CR-motility, CR-ALH, CR-viability, CR-acrosome integrity in SLP-treated mouflons were lower ($P<0.01$) than in controls when sperm was slow-frozen; CR-viability and CR-acrosome were lower than controls ($P<0.05$) when sperm was ultra-rapidly frozen. In ibexes, CR-ALH was lower for SLP-treated than untreated group ($P<0.05$). SLP treatment increased the head dimensions in ibexes ($P<0.001$). The treatment with SLP did not affect sperm head dimensions in mouflons. The results suggest that high levels of PRL negatively affect the CR of ibex and mouflon sperm.

Key words: bromocriptine, sulpiride, cryopreservation, sperm freezability, sperm morphometry, prolactin.

## 1. INTRODUCTION

The use of artificial insemination with cryopreserved sperm allows the genetic exchange between isolated wild populations that are more likely to have inbreeding problems. Nevertheless, the number of studies to improve sperm cryopreservation in wild species is still limited compared to domestic species (Watson and Holt 2001), and the fertility results are very variable (Santiago-Moreno et al., 2006a; Pradiee et al., 2017). Optimum cryoresistance is dependent not only on semen extenders (Centola et al., 1992), but also on the cooling rates used (Bóveda et al., 2020). In this sense, ultra-rapid freezing is a simple and inexpensive alternative that can be used to preserve the sperm of certain wild species in the field (O´Brien et al., 2019). The sperm sensitivity to the processes that occur during cryopreservation is related with sperm initial quality and the hormone-dependent influences that sperm are exposed from spermatogenesis to final ejaculated (Jiménez-Rabadán et al., 2016). Endocrine-related changes in the testicular germinal epithelium, and subsequently in sperm morphometric characteristics, can happen in a relatively short period of time (Martínez-Fresneda et al., 2019a). These changes in sperm head could influence on the sperm cryoresistance since water and electrolyte membrane transport during the cooling and freezing process are affected by cell size (Thurston et al., 2001).

The time in which semen is collected may also affect the freezing suitability in these species exhibiting seasonal breeding activity. Although it has long been assumed that the most favourable period to collect

semen for its cryopreservation is during the rutting season, recent studies in wild and domestic small ruminant species suggest that high testosterone levels may negatively affect sperm freezability with independency from initial sperm quality (Bóveda et al., 2021). The prolactin (PRL), which acts synergistically with testosterone to maintain specific aspects of the functions and secretory activity of male accessory sex glands (Lincoln et al., 1998), shows a strong seasonal pattern of secretion in wild ruminants, following a trend roughly parallel to photoperiod (Snyder et al., 1983; Lincoln 1998; Toledano-Díaz et al., 2012). Thus, it would be intuitive to think that those changes could also influence the sperm cryoresistance. Indeed, *in vitro* supplementation of PRL affects acrosome integrity of sperm from rams and bucks (Martínez-Fresneda et al., 2019b). In addition, PRL receptor (PRL-R) expression has been detected in the interstitial and seminiferous tubular compartments, as well as in Leydig cells, pachytene spermatocytes, and round and elongated spermatids of small ruminant testes (Jabbour and Lincoln, 1999; Lincoln et al., 2002) suggesting a putative role of PRL during spermatogenesis.

The aim of the present work was to examine how changes in the PRL concentration at key moments of the year influence sperm head dimensions and cryopreservation in wild ruminants. PRL levels were modified experimentally by administrating dopaminergic agonist and antagonist. Since sensitivity to PRL concentration-associated sperm damage could differ according to the cooling rate employed, sperm samples were cryopreserved following a conventional slow freezing and an ultra-rapid freezing protocol.

## 2. MATERIAL AND METHODS

All treatments and diluents were prepared in the laboratory using reagent-grade chemicals purchased from Sigma-Aldrich Inc.

### 2.1 Experimental design

*Experiment 1: Influence of bromocriptine (BCR) treatment on sperm head dimensions and sperm cryoresistance*

Seven ibexes and nine mouflons were divided into two groups: 1) Control group: three animals of each species were i.m. administered 1 ml of a 0.9% NaCl solution containing 60 mg of Dextran 70® twice per week from May 15 to June 15; 2) BCR group: four ibexes and six mouflons were given i.m. injections of 10 mg of BCR in the same Dextran 70®-containing vehicle twice weekly from May 15 to June 15. This administration protocol induces basal concentrations of PRL in blood plasma (Toledano-Díaz et al., 2012). Samples collection (7 per animal) was taken from 15 May to 15 August with an interval between successive collections from each animal of 15 days; one before treatment administration, two during treatment month and four post-treatment.

92 *Experiment 2: Influence of sulpiride (SLP) treatment on sperm head dimensions and cryoresistance*

93 Eight ibexes and twelve mouflons were divided in two groups: 1) Control group: three ibexes and six
94 mouflons were subcutaneously s.c. administered 2 ml of 0.9% NaCl solution containing 30 mg of tartaric
95 acid (vehicle) daily from December 15th to January 15th; 2) SLP group: five ibexes and six mouflons were
96 administered 100 mg of s.c SLP in the same vehicle daily from December 15 to January 15. Preliminary
97 studies confirmed that SLP administration increases plasma PRL. Samples collection (7 per animal) was
98 taken from 15 December to 15 March with an interval between successive collections from each animal of
99 15 days; one before treatment administration, two during treatment month and four post-treatment.

100 **2.2 Animals, ultrasound analysis and collection of samples**

101 Animals were handled according to procedures approved by the INIA Ethics Committee that
102 specifically approved the design of the current study (reference number PROEX 271/14). Animals were
103 housed in captivity at the INIA Department of Animal Reproduction. The animals were anaesthetized,
104 ultrasound examinations of the testes and sexual accessory glands were performed using real-time
105 transrectal ultrasonography and semen obtained following the TUMASG method as previously described
106 (Santiago-Moreno et al., 2013). Blood samples were collected by jugular venipuncture.

107 **2.3 Sperm quality assessment**

108 Sperm concentration was calculated before freezing using a Neubauer chamber. Sperm motility was
109 determined using a computer-aided sperm analysis (CASA) system, (Santiago-Moreno et al., 2013)
110 employing Sperm Class Analyzer® v.4.0. software (Microptic S.L., Barcelona, Spain), and membrane
111 integrity and acrosomal status analyzed by fluorescence microscopy using a fluorochrome combination of
112 propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) (Soler et
113 al., 2005). Morphological abnormalities were assessed by phase contrast microscopic examination of
114 glutaraldehyde-fixed samples. Smears were prepared for morphometric analysis as described by Esteso
115 et al. (2015). Morphometric variables were measured in fresh semen in the samples collected before the
116 treatments and during the 50 days after the end of treatment.

117 **2.4 Cryopreservation of sperm**

118 All samples were separated into two fractions and diluted 1:1 (vol/vol) to be washed twice by
119 centrifugation at 900x g for 20 minutes to retrieve the seminal plasma. The sperm was resuspended at
120 room temperature with one of two experimental extenders until reaching a final concentration of 100 x $10^6$
121 sperm/mL. The extender used for the conventional slow freezing samples was TCG for ibex and TEST for
122 mouflon (Pradiee et al., 2017). The extenders for the ultra-rapid freezing samples were the same, with the
123 glycerol substituted by 100 mM sucrose. Sperm samples were frozen (conventional slow freezing and

ultra-rapid freezing) and thawed as previously described (Bóveda et al., 2020). The response to cryopreservation in each species was illustrated by calculating a cryoresistance ratio (O'Brien et al., 2019).

**2.5 PRL analysis**

PRL plasma concentrations were determined in both seminal and blood plasma by radioimmunoassay (Gómez-Brunet and López-Sebastián, 1991). The technique sensitivity was 1.6 ng/mL and the intra-assay CV was 5% (n=10) and the inter-assay CV was 8% (n=10).

**2.6 Statistical analysis**

Data were expressed as means ± SE. The data that showed non-normal distribution as determined by the Shapiro–Wilk test were log-transformed, arcsine-transformed (percentages), or arcsinh-transformed (in case of variables including zero values) before analysis. The effects of treatments and of their interaction with the data period on sperm variables and the CR values returned by each freezing method were studied by GLM repeated measures ANOVA. All calculations were performed using Statistica for Windows v.12.0 software (StatSoft, Tulsa, OK, USA).

**3. RESULTS**

*Experiment 1: Influence of BCR treatment on sperm head dimensions and sperm cryoresistance*

Blood plasma PRL concentrations were lower in the BCR-treated animals than controls in both ibexes ($P<0.001$; 39.35 ± 14.51 ng/mL vs 119 ± 26.02 ng/mL and 22 ± 4.45 ng/mL vs 134 ± 10.92 ng/mL, in May and June, respectively) and mouflons (54.22 ± 14.78 ng/mL vs 205 ± 52.05 ng/mL and 11 ± 2.06 ng/mL vs 220 ± 50.97 ng/mL, in May and June, respectively). BCR treatment did not affect seminal plasma PRL concentrations in any species. Two months after treatment, the testicular diameter of BCR-treated mouflons was lower than untreated males ($P<0.05$; 3.43 ± 0.05 cm vs 3.75 ± 0.15 cm and $P<0.01$; 3.75 ± 0.11 cm vs 4.46 ± 0.12 cm, in July and August, respectively). The size of seminal vesicles and bulbourethral glands were not affected by BCR treatment in either ibexes or mouflons. Semen fresh of BCR-treated mouflons and ibexes showed greater sperm with morphological abnormalities than controls ($P<0.05$; 52.36 ± 6.59 % vs 24.88 ± 7.34 % in mouflons; $P<0.01$, 92.00 ± 2.84 % vs 64.75 ± 7.03 % in ibexes). During the post-treatment period, BCR-treated mouflons showed greater sperm with morphological abnormalities than controls ($P<0.01$; 61.81 ± 6.44 % vs 29.58 ± 6.91%). During the post-treatment period, the CRs for curvilinear velocity (CR-VCL), straight-line velocity (CR-VSL), and average path velocity (CR-VAP) in BCR-treated mouflons were lower ($P<0.05$) than in controls (Fig. 1) when the sperm was slow-frozen, while CR-motility and CR for morphological normal sperm BCR-treated mouflons were greater ($P<0.05$) than controls when the sperm was ultra-rapid frozen (Fig. 1). The fresh sperm cells

of the BCR-treated ibexes had a greater head width, area and perimeter than controls (P<0.001). Conversely, the sperm cells of BCR-treated mouflons showed a lower head width, area and perimeter than untreated in the post-treatment period (P<0.001) (Table 1).

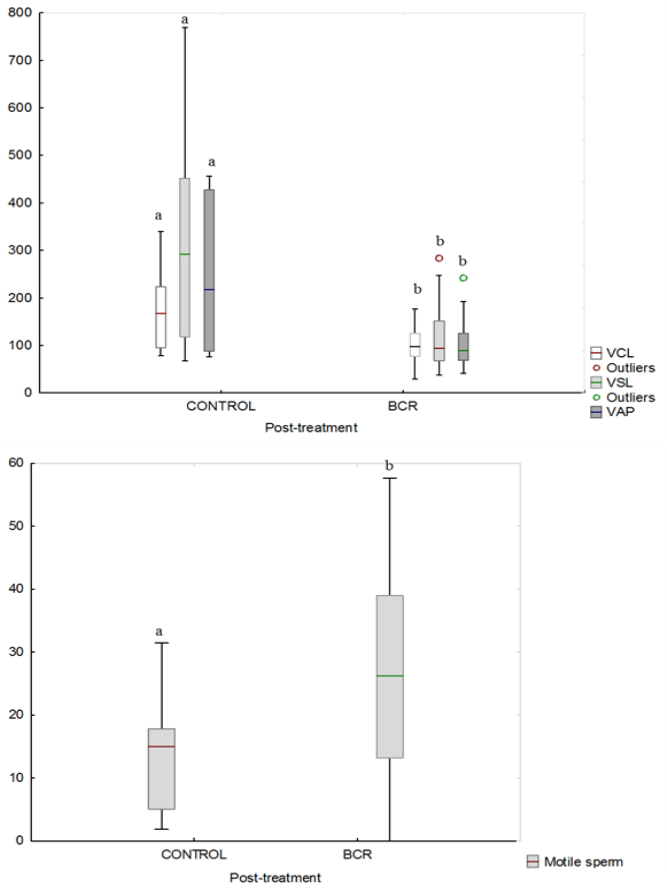


**Fig. 1.** Cryoresistance ratios for sperm variables in control and BCR-treated mouflons following the different protocols: slow-freezing (top) and ultra-rapid freezing (bottom). Different letters indicate significant differences (P<0.05) between the control and treatment group.

**Table 1.** Morphometric values (means ± SE) for fresh sperm from control and BCR-treated, before treatment and over 50 days post-treatment.

| CM | BCR before | Control before | BCR 50 days post-treatment | Control 50 days post-treatment |
|---|---|---|---|---|
| Length (µm) | 8.24 ± 0.03 | 8.02 ± 0.04 | 7.82 ± 0.03 | 7.81 ± 0.04 |
| Width (µm) | 4.22 ± 0.01 | 4.25 ± 0.01 | 4.14 ± 0.01[a] | 3.99 ± 0.01[b] |
| Area (µm²) | 28.79 ± 0.15 | 28.27 ± 0.18 | 26.81 ± 0.14[a] | 25.84 ± 0.18[b] |
| Perímeter (µm) | 21.59 ± 0.06 | 21.23 ± 0.08 | 20.71 ± 0.06[a] | 20.45 ± 0.08[b] |
| Acrosome (%) | 57.24 ± 0.14 | 55.80 ± 0.18 | 56.72 ± 0.15[b] | 57.47 ± 0.18[a] |
| **MU** | **BCR before** | **Control before** | **BCR 50 days post-treatment** | **Control 50 days post-treatment** |
| Length (µm) | 9.02 ± 0.01 | 8.94 ± 0.02 | 8.61 ± 0.02 | 8.67 ± 0.02 |

| | | | | |
|---|---|---|---|---|
| Width (µm) | 5.01 ± 0.01 | 5.04 ± 0.01 | 4.78 ± 0.01[b] | 4.96 ± 0.01[a] |
| Area (µm$^2$) | 37.17 ± 0.10 | 37.09 ± 0.12 | 33.99 ± 0.13[b] | 35.47 ± 0.13[a] |
| Perímeter (µm) | 24.03 ± 0.03 | 23.94 ± 0.04 | 22.98 ± 0.05[b] | 23.35 ± 0.05[a] |
| Acrosome (%) | 55.60 ± 0.10 | 55.84 ± 0.13 | 55.43 ± 0.09 | 55.60 ± 0.12 |

Different letters indicate significant differences (P<0.05) between control and treatment groups post-treatment (lower case letters). Asterisks show significant differences (P<0.05) between pre-treatment and 50 days post-treatment. Abbreviations: BCR, bromocriptine. Acrosome (%): percentage of the head occupied by the acrosome.

*Experiment 2: Influence of SLP treatment during the rutting season on sperm cryoresistance*

Blood plasma PRL levels increased after SLP administration but basal concentrations were reached by 24h post-administration in both species. No differences were seen in seminal plasma PRL concentration between SLP-treated and controls in both species. Two weeks after the treatment, testicular diameter for SLP-treated ibexes was lower than controls (P<0.05; 2.34 ± 0.14 cm vs 2.82 ± 0.05 cm respectively), while seminal vesicles for SLP-treated mouflons were greater than controls (P<0.001; 0.40 ± 0.02 cm$^2$ vs 0.30 ± 0.01 cm$^2$). The semen fresh of SLP-treated mouflons showed values of VCL and ALH greater than untreated ones during the treatment period (P<0.01; 77.81 ± 4.04 vs 56.74 ± 5.46 for VCL and 2.86 ± 0.15 vs 2.12 ± 0.15 for ALH respectively). No differences were seen for semen fresh in ibexes. During the treatment period, SLP-treated ibexes showed CR for normal sperm lower than untreated males when semen was slow and ultra-rapidly frozen (P<0.05) (Fig. 2). CR-motility, CR-ALH, CR-viability, CR-acrosome integrity in SLP-treated mouflons were lower (P<0.01) than in controls when semen was slow-frozen (Fig. 3); CR-viability and CR-acrosome were lower than controls (P<0.05) when semen was ultra-rapidly frozen (Fig. 3). In the post-treatment period, CR-ALH in SLP-treated mouflons and CR-acrosome integrity were lower (P<0.05) than controls when sperm was slow-frozen (Fig. 3). CR-ALH was lower for SLP-treated ibexes than untreated (P<0.05) (Fig. 2). SLP-treated ibexes showed greater head dimensions than controls (P<0.001). The treatment with SLP did not affect sperm head dimensions in mouflons (Table 2).

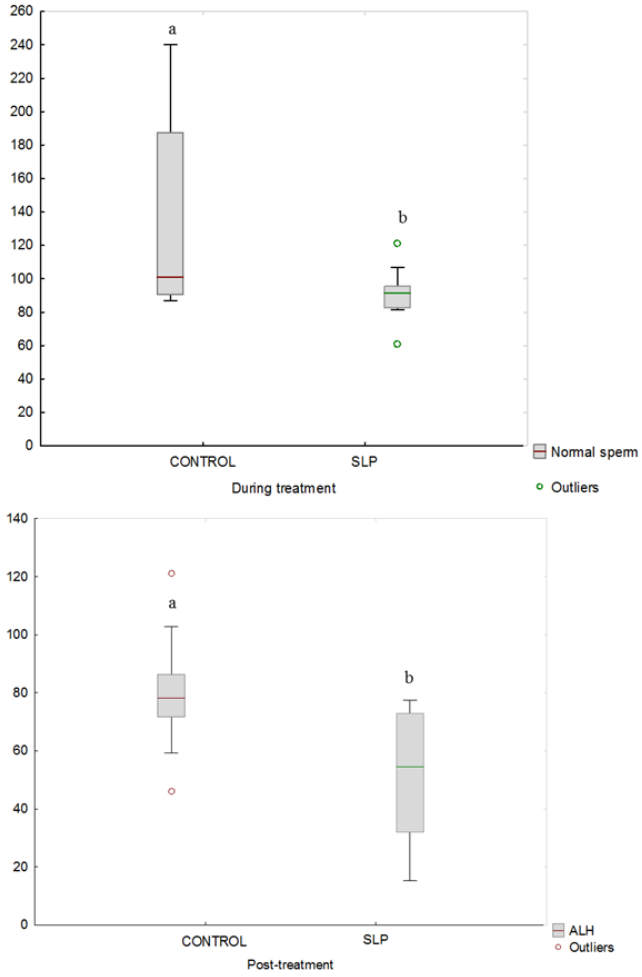


**Fig. 2.** Cryoresistance ratios for sperm variables in control and SLP-treated ibexes following the different protocols: slow-freezing (top) and ultra-rapid freezing (bottom). Different letters indicate significant differences (P<0.05) between the control and treatment group.

**Table 2.** Morphometric values (means ± SE) for fresh sperm from control and SLP-treated, before treatment and over 50 days post-treatment.

| CM | SLP before | Control before | SLP 50 days post-treatment | Control 50 days post-treatment |
|---|---|---|---|---|
| Length (µm) | 8.40 ± 0.02 | 8.50 ± 0.03 | 8.37 ± 0.02[a] | 8.24 ± 0.02[b] |
| Width (µm) | 4.20 ± 0.01 | 4.23 ± 0.01 | 4.25 ± 0.01[a] | 4.14 ± 0.01[b] |
| Area (µm$^2$) | 29.21 ± 0.10 | 29.75 ± 0.16 | 29.46 ± 0.10[a] | 28.29 ± 0.11[b] |
| Perímeter (µm) | 21.82 ± 0.04 | 22.02 ± 0.06 | 21.83 ± 0.04[a] | 21.47 ± 0.04[b] |
| Acrosome (%) | 55.72 ± 0.13 | 56.72 ± 0.16 | 56.67 ± 0.11 | 56.80 ± 0.13 |
| **MU** | **SLP before** | **Control before** | **SLP 50 days post-treatment** | **Control 50 days post-treatment** |
| Length (µm) | 8.96 ± 0.01 | 8.98 ± 0.01 | 8.94 ± 0.01 | 8.90 ± 0.01 |
| Width (µm) | 4.91 ± 0.01 | 4.91 ± 0.01 | 4.89 ± 0.00 | 4.91 ± 0.01 |
| Area (µm$^2$) | 36.24 ± 0.08 | 36.35 ± 0.08 | 36.03 ± 0.07 | 35.94 ± 0.07 |
| Perímeter (µm) | 23.79 ± 0.03 | 23.85 ± 0.03 | 23.70 ± 0.02 | 23.64 ± 0.02 |
| Acrosome (%) | 55.28 ± 0.08 | 55.26 ± 0.08 | 55.45 ± 0.08[b] | 55.83 ± 0.08[a] |

Different letters indicate significant differences (P<0.05) between control and treatment groups post-treatment (lower case letters). Asterisks show significant differences (P<0.05) between pre-treatment and 50 days post-treatment. Abbreviations: SLP, sulpiride. Acrosome (%): percentage of the head occupied by the acrosome.

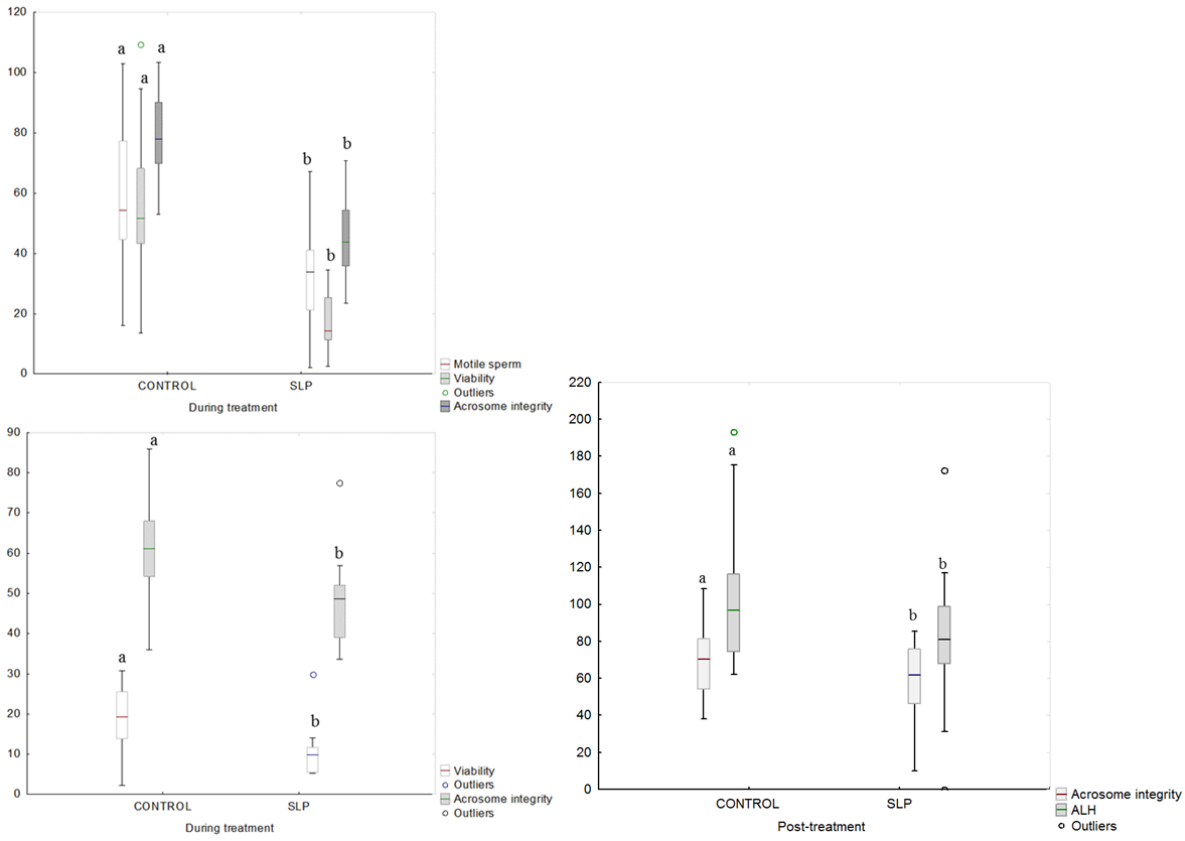


**Fig. 3.** Cryoresistance ratios for sperm variables in control and SLP-treated mouflons following the different protocols: slow-freezing (top) and ultra-rapid freezing (bottom) during treatment (Left figures), and post-treatment (Right figure). Different letters indicate significant differences (P<0.05) between the control and treatment group.

## 4. DISCUSSION

BCR and SLP treatments modified the blood plasma PRL concentration as expected in both species (Toledano et al., 2012; Lincoln et al., 2003) - modifications that were associated with changes in sperm cryoresistance. BCR treatment produced a variable effect on sperm cryoresistance (decreasing or increasing sperm motility variables), but SLP treatment always induced a negative influence on sperm freezability.

In fresh samples, the decreasing of PRL by BCR treatment increased the sperm with morphological abnormalities. In the same way, the presence of increased levels of PRL by SLP improved certain kinetics variables, such as VCL and ALH, and the percentage of sperm with normal morphology. PRL appears to act directly, and also synergistically, with testosterone to maintain specific aspects of the functions and secretory activity of male accessory sex glands (Hair et al., 2002; Santiago-Moreno et al., 2006b). It's known that an endocrine balance between hormones produced in the pituitary and in the testis have an effect on spermatogenesis and spermiogenesis processes (Xu et al., 1991). PRL acts in the Leydig cells

of the testis, to increase their responsiveness to LH to restore spermatogenesis in rats (Bartke, 1971), and has a direct effect on sperm cell metabolism, motility and fertilizing capacity (Fukuda et al., 1989).

The influence of SLP treatment on cryoresistance during the period of treatment in mouflons and ibex suggest an effect at epididymal level, where PRL is involved in sperm maturation (Reddy et al., 1985). Although ultra-rapid cooling rates usually produce stronger ultrastructural damages on sperm cell than conventional slow cooling rates (Bóveda et al., 2020), our finding revealed that the decreasing of CR after SLP treatment was variable for different sperm parameters, according the methods of freezing: slow or ultra-rapid. On the other hand, the decreasing of CR for ALH and acrosome, in the post-treatment period, suggest an effect of PRL variations through spermatogenic cycle in both species. The effect of endocrine changes (e.g. testosterone variations) on sperm freezability by an indirect influence during spermatogenic cycle has been previously reported in mouflons (Martinez-Fresneda et al., 2019a). Our data confirm that despite high levels of PRL may be associated with an appropriated sperm function, it negatively affects the sperm CR (Flores-Gil et al., 2021). The decreasing of CR-acrosome integrity after SLP treatment is in agreement with previous findings in rams and bucks, in which *in vitro* incubation with PRL decreased the post-thaw acrosome integrity (Martínez-Fresneda et al., 2019b).

Unlike SLP treatment, the BCR treatment returned a variable effect on sperm freezability, for instance decreasing the kinetic variables (VCL, VSL and VAP) and improving the CR for total motile sperm. This may be explained because along with indirect effect decreasing on PRL secretion, the BCR could affect the dopaminergic signaling in capacitation and sperm motility. Indeed, dopamine type 2 receptors (DRD2) are present in a wide range of mammalian sperm (Otth et al., 2007; Ramírez et al., 2009). Bromocriptine and low-concentration dopamine increased total and progressive motility of boar sperm; conversely bromocriptine and high dopamine levels increased tyrosine phosphorylation during the capacitation period (Ramírez et al., 2009). During sperm capacitation, sperm cell suffers some modification on permeability and fluidity of membrane, ions transport, which could decrease the CR on sperm cell in function of cooling rate applied. On the other hand, human sperm directly exposed to BCR had a decrease of motility and of some kinetic variables (Chenette et al., 1991).

Our findings showed that both treatments affected the sperm head dimensions in fresh samples about 50 days post-treatment. BCR treatment increased the head sperm size in ibexes; conversely, BCR treatment decreased the head sperm size in mouflons. Similarly, the BCR treatment in domestic rams also reduced the sperm head dimensions (Flores-Gil et al., 2021). Differences in sperm head dimension may influence on sperm water volume, membrane permeability to water and cryoprotectant concentration, and thus sperm freezability (Curry, 2000). It has been reported that the sperm cells of less cryopreservation-sensitive species are smaller (Garde et al., 2003). The decrease on sperm head sizes could explain the better response obtained for CR-motility after ultra-rapid freezing. In turn, SLP treatment increased the sperm head size in both species, which appears to be associated with a decreased cryotolerance.

PRL may play a physiological role in the growth of the testes and sex accessories in some species (Jabbour et al., 1998; Howell-Skalla et al., 2002; Santiago-Moreno et al., 2013) which may explain the increase of seminal vesicles size after SLP treatment of mouflons, and the decrease of testicular size after BCR treatment. Variations in the dimensions of these glands are accompanied with changes in their functional activity that in turn might affect sperm and their tolerance to cryopreservation process.

In conclusion, this is the first study in wild ruminants where the role of PRL plasma concentrations on sperm cryopreservation response was evaluated. The results suggest that high levels of PRL negatively affect the CR of ibex and mouflon sperm. The specie-specific response to freezing-thawing process after either BCR or SLP treatments may be due to differences in dopaminergic receptor expression in sperm (Otth et al., 2007), along with chemical and physical differences, in plasma membrane composition (Ladha 1998).

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**REFERENCES**

1. Watson, P., & Holt, W. V. (2001). Cryobanking the genetic resource: Wildlife conservation for the future?. CRC Press.
2. Santiago-Moreno, J., Toledano-Díaz, A., Pulido-Pastor, A., Gómez-Brunet, A., & López-Sebastián, A. (2006a). Birth of live Spanish ibex (*Capra pyrenaica hispanica*) derived from artificial insemination with epididymal spermatozoa retrieved after death. *Theriogenology*, 66(2), 283-291.
3. Pradiee, J., Esteso, M. C., Castaño, C., Toledano-Díaz, A., Lopez-Sebastián, A., Guerra, R., & Santiago-Moreno, J. (2017). Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. *Andrologia*, 49(3), e12629.
4. Centola, G. M., Raubertas, R. F., & Mattox, J. H. (1992). Cryopreservation of human semen: comparison of cryopreservatives, sources of variability, and prediction of post-thaw survival. *Journal of Andrology*, 13(3), 283-288.
5. Bóveda, P., Toledano-Díaz, A., Castaño, C., Esteso, M. C., López-Sebastián, A., Rizos, D., ... & Santiago-Moreno, J. (2020). Ultra-rapid cooling of ibex sperm by spheres method does not

induce a vitreous extracellular state and increases the membrane damages. *Plos one*, 15(1), e0227946.

6. O'Brien, E., Esteso, M. C., Castaño, C., Toledano-Díaz, A., Bóveda, P., Martínez-Fresneda, L., ... & Santiago-Moreno, J. (2019). Effectiveness of ultra-rapid cryopreservation of sperm from endangered species, examined by morphometric means. *Theriogenology*, 129, 160-167.
7. Jiménez-Rabadán, P., Soler, A. J., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Iniesta-Cuerda, M., ... & Garde, J. J. (2016). Influence of semen collection method on sperm cryoresistance in small ruminants. *Animal reproduction science*, 167, 103-108.
8. Martínez-Fresneda, L., O'Brien, E., Velázquez, R., Toledano-Díaz, A., Martínez-Cáceres, C. M., Tesfaye, D., ... & Santiago-Moreno, J. (2019). Seasonal variation in sperm freezability associated with changes in testicular germinal epithelium in domestic (*Ovis aries*) and wild (*Ovis musimon*) sheep. *Reproduction, Fertility and Development*, 31(10), 1545-1557.
9. Thurston, L. M., Watson, P. F., Mileham, A. J., & Holt, W. V. (2001). Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *Journal of Androl*ogy, 22(3), 382-394.
10. Bóveda, P., Esteso, M. C., Velázquez, R., Castaño, C., Toledano-Díaz, A., López-Sebastián, A., ... & Santiago-Moreno, J. (2021). Influence of circulating testosterone concentration on sperm cryoresistance: The ibex as an experimental model. *Andrology*, 00, 1-12.
11. Lincoln, G. A. (1998). Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*Ovis musimon*). *Animal Reproduction Science*, 53(1-4), 87-105.
12. Snyder, D. L., Cowan, R. L., Hagen, D. R., & Schanbacher, B. D. (1983). Effect of pinealectomy on seasonal changes in antler growth and concentrations of testosterone and prolactin in white-tailed deer. *Biology of reproduction*, 29(1), 63-71.
13. Toledano-Díaz, A., Santiago-Moreno, J., Gómez-Brunet, A., & López-Sebastián, A. (2012). Prolactin inhibition does not influence horn growth in two wild caprinae species: European mouflon (*Ovis orientalis musimon*) and Iberian ibex (*Capra pyrenaica*). *European journal of wildlife research*, 58(5), 803-810.
14. Martínez-Fresneda, L., O'Brien, E., Sebastián, A. L., Velázquez, R., Toledano-Díaz, A., Tesfaye, D., ... & Santiago-Moreno, J. (2020). In vitro supplementation of testosterone or prolactin affects spermatozoa freezability in small ruminants. *Domestic animal endocrinology*, 72, 106372.
15. Jabbour, H. N., & Lincoln, G. A. (1999). Prolactin receptor expression in the testis of the ram: localisation, functional activation and the influence of gonadotrophins. Molecular and cellular endocrinology, 148(1-2), 151-161.
16. Lincoln, G. (2002). Melatonin modulation of prolactin and gonadotrophin secretion. *Melatonin after Four Decades*, 137-153.

17. Santiago-Moreno, J., Castaño, C., Toledano-Díaz, A., Esteso, M. C., López-Sebastián, A., Guerra, R., ... & Hildebrandt, T. B. (2013). Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. *Theriogenology*, 79(2), 383-391.
18. Soler, A. J., Esteso, M. C., Fernández-Santos, M. R., & Garde, J. J. (2005). Characteristics of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa cryopreserved after storage at 5 C in the epididymis for several days. *Theriogenology*, 64(7), 1503-1517.
19. Esteso, M. C., Rodríguez, E., Toledano-Díaz, A., Castaño, C., Pradiee, J., López-Sebastián, A., & Santiago-Moreno, J. (2015). Descriptive analysis of sperm head morphometry in Iberian ibex (*Capra pyrenaica*): Optimum sampling procedure and staining methods using Sperm-Class Analyzer®. *Animal reproduction science*, 155, 42-49.
20. Gómez-Brunet, A. G., & López-Sebastián, A. L. (1991). Effect of season on plasma concentrations of prolactin and cortisol in pregnant, non-pregnant and lactating ewes. *Animal Reproduction Science*, 26(3-4), 251-268.
21. Lincoln, G. A., Andersson, H., & Clarke, I. J. (2003). Prolactin cycles in sheep under constant photoperiod: evidence that photorefractoriness develops within the pituitary gland independently of the prolactin output signal. *Biology of reproduction*, 69(4), 1416-1423.
22. Hair, W. M., Gubbay, O., Jabbour, H. N., & Lincoln, G. A. (2002). Prolactin receptor expression in human testis and accessory tissues: localization and function. *Molecular human reproduction*, 8(7), 606-611.
23. Santiago-Moreno, J., Gómez-Brunet, A., Toledano-Díaz, A., Picazo, R., Gonzalez-Bulnes, A., & López-Sebastián, A. (2006). Seasonal endocrine changes and breeding activity in Mediterranean wild ruminants. *Reproduction in Domestic Animals*, 41, 72-81.
24. Xu, Z. Z., McDonald, M. F., McCutcheon, S. N., & Blair, H. T. (1991). Seasonal variation in testis size, gonadotrophin secretion and pituitary responsiveness to GnRH in rams of two breeds differing in time of onset of the breeding season. *Animal Reproduction Science*, 26(3-4), 281-292.
25. Bartke, A. (1971). Effects of prolactin and luteinizing hormone on the cholesterol stores in the mouse testis. *Journal of Endocrinology*, 49(2), 317-324. Fukuda A, Mori C, Hashimoto H, Noda Y, Mori T, Hoshino K. Effects of prolactin during preincubation of mouse spermatozoa on fertilizing capacity in vitro. J In Vitro Fert Embryo Transf 1989;6:92–7.
26. Reddy, Y. D., Reddy, K. V., & Govindappa, S. (1985). Effect of prolactin and bromocriptine administration on epididymal function—a biochemical study in rats. *Indian J Physiol Pharmacol*, 29, 234-238.
27. Flores-Gil, V. N., Toledano-Díaz, A., Velázquez, R., Oteo, M., López-Sebastián, A., & Santiago-Moreno, J. (2021). Role of changes in plasma prolactin concentrations on ram and buck sperm cryoresistance. *Domestic Animal Endocrinology*, 106624.

28. Otth, C., Torres, M., Ramírez, A., Fernandez, J. C., Castro, M., Rauch, M. C., ... & Concha, I. I. (2007). Novel identification of peripheral dopaminergic D2 receptor in male germ cells. *Journal of cellular biochemistry*, 100(1), 141-150.
29. Ramírez, A. R., Castro, M. A., Angulo, C., Ramió, L., Rivera, M. M., Torres, M., ... & Concha, I. I. (2009). The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility. *Biology of reproduction*, 80(4), 753-761.
30. Chenette, P. E., Siegel, M. S., Vermesh, M., & Kletzky, O. A. (1991). Effect of bromocriptine on sperm function in vitro and in vivo. *Obstetrics and gynecology*, 77(6), 935-938.
31. Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Reviews of reproduction*, 5(1), 46-52.
32. Garde, J. J., Soler, A. J., Cassinello, J., Crespo, C., Malo, A. F., Espeso, G., ... & Roldan, E. R. S. (2003). Sperm cryopreservation in three species of endangered gazelles (*Gazella cuvieri, G. dama mhorr,* and *G. dorcas neglecta*). *Biology of reproduction*, 69(2), 602-611.
33. Jabbour, H. N., Clarke, L. A., McNeilly, A. S., Edery, M., & Kelly, P. A. (1998). Is prolactin a gonadotrophic hormone in red deer (*Cervus elaphus*)? Pattern of expression of the prolactin receptor gene in the testis and epididymis. Journal of molecular endocrinology, 20(2), 175-182.
34. Howell-Skalla, L. A., Cattet, M. R., Ramsay, M. A., & Bahr, J. M. (2002). Seasonal changes in testicular size and serum LH, prolactin and testosterone concentrations in male polar bears (*Ursus maritimus*). *Reproduction*, 123(5), 729-733.
35. Ladha, S. (1998). Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. *The Journal of membrane biology*, 165(1), 1-10.

# DISCUSIÓN

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Los resultados de la presente tesis doctoral muestran que la congelación ultra-rápida puede ser un método alternativo de criopreservación de espermatozoides de rumiantes silvestres. Si bien, la congelación convencional muestra, en general, mejores resultados de calidad espermática y menos intensidad de daños ultra-estructurales tras la descongelación, la congelación ultra-rápida puede ser una alternativa en aquellas condiciones de campo en las que se requiera un procedimiento de criopreservación rápido, fácil de ejecutar y a bajo costo. Los resultados también ponen de manifiesto una influencia del estatus endocrino en la crio-tolerancia de los espermatozoides de rumiantes silvestres, que explicaría la variable respuesta a la congelación en función de la época del año.

Uno de los objetivos del primer trabajo era confirmar la existencia de vitrificación en el medio extracelular, cuando se usa el método de criopreservación ultra-rápida. La consecución de un estado vítreo a nivel intra- y extra- celular depende de la interacción entre la velocidad de enfriamiento, la viscosidad y el volumen de la solución (Shaw y Jones, 2003). Por otro lado, se ha asumido que los posibles cristales que puedan llegar a formarse en el medio extracelular, durante un enfriamiento ultra-rápido de volúmenes pequeños, serían despreciables y no llegarían a producir un daño sobre las células debido a su pequeño tamaño (Isachenko et al., 2003). Sin embargo, los presentes resultados indican que el método de congelación ultra-rápida en pellets, utilizado en nuestro estudio, no previene la formación de cristales de hielo en el medio extracelular. No obstante, es necesario tener en cuenta que hay poca información sobre esta técnica, y que el volumen usado en este caso (50 µl), ha sido mayor que el usado en otros estudios que llevan a cabo una vitrificación cinética (20 µl) (Isachenko et al., 2004). La sucrosa es el crioprotector no permeable utilizado para la congelación ultra-rápida. Se ha sugerido que la sucrosa ralentiza la fase de nucleación del hielo (Caldwell et al., 1992; Cook y Hartel, 2010), por lo que puede afectar a la forma de los propios cristales. Esto podría explicar el hecho de que, tras la congelación ultra-rápida, los cristales observados fueron más pequeños y más estriados que los observados tras la congelación convencional. Este patrón de formación de hielo podría favorecer un contacto más directo del hielo con las células espermáticas, induciendo un mayor daño en las mismas.

El estudio de muestras espermáticas a través de microscopía electrónica de barrido y transmisión (TEM), nos ha permitido concluir que no hay evidencia de hielo intracelular en la cabeza de los espermatozoides para ambos métodos de congelación. Estos resultados coinciden con diferentes estudios en los cuales, tras la aplicación de diferentes rangos de enfriamiento en la congelación, no se observa la formación de hielo intracelular, sugiriendo que la vitrificación intracelular siempre ocurre (Morris 2006; Morris et al., 2007, 2012), a menos a nivel de la cabeza espermática. De hecho, algunos autores indican que el hielo intracelular no se formaría, incluso cuando se usan rampas de congelación lentas, en las cuales, teóricamente, debería ser más probable (Ekwall et al., 2007). Los espermatozoides sometidos a una congelación ultra-rápida sufrieron un mayor daño a nivel de plasmalema, membrana del acrosoma

y membrana mitocondrial, junto a unos valores de motilidad espermática más bajos que la congelación convencional. Esto puede estar relacionado con el rápido enfriamiento al que se someten las células, lo que favorecería un mayor daño por shock frío, asociado con los cambios de estado de las membranas celulares (gel o fluido) durante la temperatura de transición (Drobnis et al., 1993). Junto a los daños a nivel de membrana, en los espermatozoides criopreservados mediante congelación ultra-rápida se apreciaron cambios sustanciales en las dimensiones de la cabeza, por variaciones en el volumen celular durante los procesos de congelación y descongelación (Esteso et al., 2003). Los cambios que se producen a nivel morfométrico están relacionados directamente con el estrés osmótico al que se someten las células, con las interacciones de agua y soluto tras la cristalización (Aboagla y Terada, 2004). La TEM ha mostrado que algunas formaciones de hielo intracelular no se pueden evitar fuera de la cabeza del espermatozoide. De hecho, ciertos cambios estructurales sugieren la formación de cristales en pieza intermedia y colas, coincidiendo con otros trabajos (Courtens y Paquignon, 1985; Sherman y Liu, 1988). El descenso en la motilidad espermática puede estar relacionado directamente con los daños que se producen a nivel de membranas mitocondriales (Piomboni et al., 2012). Mediante el análisis de fluorescencia a través de Mitotracker Green FM (MITO), se pudo determinar que estos daños fueron mayores en aquellas células que fueron sometidas a un proceso de congelación ultra-rápida. La evaluación mediante TEM ha permitido determinar que la pieza intermedia presentaba un volumen inferior tras el método de congelación ultra-rápida al observado tras el método de congelación lenta, lo que sugiere la incapacidad de las células para volver al volumen inicial tras sufrir un choque osmótico, quizás debido a un daño de membrana plasmática irreversible (González-Fernández et al., 2012).

A pesar de la mayor intensidad en los daños ultra-estructurales y funcionales, la congelación ultra-rápida permite obtener un cierto porcentaje de espermatozoides funcionales, lo que la sitúa como una alternativa prometedora y práctica a los métodos convencionales, principalmente cuando se trabaja con especies silvestres en condiciones de campo y con ciertas limitaciones a nivel de equipo de laboratorio.

La criopreservación de espermatozoides en especies silvestres está limitada por la dificultad en la obtención de muestras en condiciones de campo y la congelación posterior de las mismas. Los resultados del segundo trabajo de la presente tesis muestran que la congelación ultra-rápida de espermatozoides epididimarios recogidos *post-mortem* puede ser un método alternativo de criopreservación en condiciones de campo. La literatura contiene pocos trabajos sobre congelación ultra-rápida de células espermáticas de rumiantes silvestres (Pradiee et al., 2015). La adición de ciertas concentraciones bajas de crioprotectores no-permeables como la albúmina, y osmoprotectores como la sucrosa, han sido estudiados para la congelación ultra-rápida con una mejora en la criorresistencia de células espermáticas en humanos (Isachenko et al., 2008), perro (Sánchez et al., 2011) y macho montés (Pradiee et al., 2015, 2017). Las células espermáticas epididimarias son más crio-resistentes que los espermatozoides eyaculados (Martínez-Fresneda et al., 2019), encontrándose diferencias en el proteoma espermático

(Martínez Fresneda et al., 2021) y en la propia composición de membrana (Eddy and O'Brien, 1994; Jones, 1998; Varisli et al., 2009). Todo esto influye en la variable respuesta a los procesos de congelación-descongelación y en la necesidad de adecuar protocolos de criopreservación específicos, por ejemplo, en términos del tiempo de equilibrado requerido (Rath y Niemann, 1997). La presente tesis ha permitido determinar que, para muestras espermáticas obtenidas *post-mortem* en muflón y gamo, el proceso de criopreservación provoca una reducción del tamaño de la cabeza de los espermatozoides en ambas especies, lo que está en consonancia con lo apuntado para otras especies de rumiantes domésticas y silvestres (Hidalgo et al., 2007, Esteso, 2006, Pradiee et al., 2016). Sin embargo, comparando ambos métodos de congelación, en el caso del gamo, el área de la cabeza del espermatozoide ha sido inferior tras la congelación ultra-rápida que tras la congelación convencional lo que sugiere una mayor sensibilidad frente al procedimiento ultra-rápido.

La limitada efectividad de la criopreservación de espermatozoides en especies de ungulados silvestres, así como las variaciones estacionales en su criotolerancia, nos ha llevado a plantear una posible influencia de los cambios en el estatus endocrino, que acompañan a la estacionalidad reproductiva, sobre la congelación espermática. Una profundización de los conocimientos sobre los cambios fisiológicos que puedan afectar de manera directa al espermatozoide y al medio en el cual se desarrolla (plasma seminal y fluido epididimario) durante el ciclo reproductivo anual, es fundamental para llegar a entender y explicar las causas de los bajos rendimientos de fertilidad con material espermático criopreservado en pequeños rumiantes silvestres. El periodo que ofrece los mejores resultados de calidad espermática tras el proceso de congelación/descongelación, no es, como podría esperarse, aquel que coincide con las mayores concentraciones de testosterona y óptima calidad espermática, al inicio de la estación sexual, sino al final de la misma, cuando los niveles ya han disminuido sensiblemente, aunque los espermatozoides derivan de un ciclo espermatogénico previo con óptimas condiciones de niveles de andrógenos (Coloma et al., 2010). Estas observaciones plantearon la hipótesis de que la secreción altos niveles de testosterona puede afectar negativamente en la criorresistencia espermática. Para testar dicha hipótesis se utilizó el macho montés como modelo animal. El tratamiento con acetato de ciproterona (CA) indujo niveles de testosterona similares a los encontrados fuera del periodo reproductivo (Coloma et al., 2011), mientras que la administración de propionato de testosterona (TP) determinó concentraciones fisiológicas altas de testosterona, similar a las observadas durante el periodo de pre-celo (Santiago-Moreno et al., 2012). La disminución de las concentraciones de testosterona en plasma sanguíneo se asoció a un incremento de la criorresistencia, mientras que el incremento de las concentraciones plasmáticas de testosterona afectó negativamente a la criorresistencia de diferentes variables espermáticas. Es interesante destacar que la mayoría de las diferencias encontradas en la criorresistencia de las distintas variables espermáticas han sido observadas coincidiendo con el periodo de aplicación de los respectivos tratamientos con CA y TP. Considerando que la duración estimada del ciclo espermatogénico en esta especie, según estudios en la cabra doméstica, sería de unos 48 días

(França et al., 1999), se podría deducir que la influencia de las variaciones de la testosterona tendrían lugar, principalmente, durante el paso de los espermatozoides a través del epidídimo, en concreto durante la maduración final en la cola del mismo, y durante el corto periodo en el que los espermatozoides están expuestos a las secreciones de las vesículas seminales durante la eyaculación. De hecho, la actividad absorbente y secretora del epitelio del epidídimo está regulada por andrógenos (Brooks, 1983). La manipulación de las concentraciones plasmáticas de testosterona determinó cambios en las variables morfométricas de la cabeza espermática. Concretamente, el tratamiento con CA determinó una disminución del tamaño de la cabeza espermática, lo que sugiere que el ciclo espermatogénico podría haberse visto afectado. Además, tras el tratamiento con TP, el tamaño de las cabezas espermáticas se incrementaba, de forma similar a lo reportado en cabras domésticas (Flores-Gil et al., 2020). Estos resultados sugieren que las altas concentraciones de testosterona inducen un incremento de las dimensiones de la cabeza espermática. El tamaño de la cabeza espermática se ha relacionado con la susceptibilidad frente a procesos de congelación y descongelación, sin embargo, los resultados obtenidos no han permitido establecer una clara asociación entre el tamaño de la cabeza y la criorresistencia espermática, de forma similar a lo reportado previamente en otras especies silvestres (O'Brien et al., 2019). Este tercer trabajo dentro de la presente tesis, nos ha permitido determinar que la testosterona parece tener una influencia negativa sobre la criorresistencia espermática, independientemente del tamaño de la cabeza, lo que explicaría las variaciones estacionales encontradas en la efectividad de la congelación espermática de algunas especies. Los resultados proporcionan información relevante respecto al papel que juega el estatus endocrino en la criorresistencia espermática, y abre puertas al posible uso de la testosterona como marcador biológico para predecir la respuesta de los espermatozoides frente a procesos de congelación-descongelación, así como posibles vías de optimización de las técnicas de criopreservación mediante manipulación de los niveles de testosterona.

Finalmente, el papel relevante que tiene la PRL en la función reproductiva del macho, nos hizo plantear, al igual que con la testosterona, la posible influencia de sus variaciones estacionales sobre la congelación espermática. Para ello, en el cuarto trabajo se utilizó a la cabra montés y al muflón como modelos animales. El tratamiento con BCR disminuyó los niveles plasmáticos de PRL fuera de la época reproductiva (periodo en el cual, los niveles se encuentran elevados), mientras que el tratamiento de SLP los incrementó durante la época reproductiva (periodo en el cual los niveles se encuentra basales). La disminución de los niveles de PRL se asoció a un efecto variable sobre la criorresistencia espermática, mientras que el incremento en las concentraciones plasmáticas de PRL provocó una influencia negativa sobre la congelación de los espermatozoides. La PRL parece actuar directamente, y también sinérgicamente, con la testosterona para mantener aspectos específicos en las funciones y la actividad secretora de las glándulas sexuales accesorios masculinas (Hair et al., 2002; Santiago-Moreno et al., 2006b). La influencia en la criorresistencia durante el periodo de administración de SLP, sugiere para ambas especies que, el incremento de las concentraciones plasmáticas de PRL tiene un efecto a nivel de

epidídimo, donde esta hormona está implicada en la maduración espermática (Reddy et al., 1985). A pesar de que los altos niveles de PRL deberían estar asociados a una mejor función espermática, los resultados mostraron una influencia negativa sobre la criorresistencia espermática coincidiendo con otro estudio realizado en rumiantes domésticos (Flores-Gil et al., 2021). A su vez, la respuesta al tratamiento con SLP fue variable para los distintos parámetros espermáticos en ambos métodos de criopreservación, a pesar de que la congelación ultra-rápida produzca fuertes daños ultraestructurales en comparación con la congelación convencional (Bóveda et al., 2020). Sin embargo, la BCR presentó un efecto variable sobre la criorresistencia, principalmente disminuyendo las variables cinéticas, pero aumentando la velocidad, mostrando su posible implicación en la capacitación y la motilidad espermática (Ramírez et al., 2009), debido a los cambios que se producen en la permeabilidad y fluidez de membrana. Por otro lado, ambos tratamientos tuvieron un efecto sobre las dimensiones de la cabeza del espermatozoide en las muestras en fresco 50 días después de los tratamientos, con una respuesta variable dependiendo de la especie para la BCR, que disminuía el tamaño en el caso del muflón, mientras que la SLP incrementó las dimensiones de la cabeza en ambas especies, pudiendo estar relacionado con una disminución de la criotolerancia. Por último, las variaciones en el tamaño de las vesículas seminales según el tratamiento administrado, demostrarían la importancia fisiológica de la PRL en el crecimiento de las mismas, y su relación con la actividad reproductiva, la cual podría relacionarse con la tolerancia frente a procesos de criopreservación. Este último trabajo ha permitido determinar que la PRL parece tener un efecto negativo sobre la criorresistencia espermática, siendo el primer estudio realizado acerca del papel que juegan las variaciones en las concentraciones plasmáticas de PRL en la respuesta a la criopreservación en especies silvestres. Estos resultados, junto a los obtenidos con la testosterona ponen de relieve el importante papel de ambas hormonas en la variación estacional de la criorresistencia espermática.

# CONCLUSIONES

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**1.** La congelación ultra-rápida no produce un estado vítreo en el medio extracelular, pero la morfología y tamaño de los cristales extracelulares varía en comparación con la congelación convencional.

**2.** La congelación ultra-rápida determina mayores daños ultra-estructurales y funcionales en la célula espermática que la congelación convencional.

**3.** La congelación ultra-rápida de espermatozoides epididimarios recogidos *post-mortem* puede ser un método alternativo de criopreservación en condiciones de campo que requieran un procedimiento rápido y sencillo, a bajo coste.

**4.** Las concentraciones elevadas de testosterona plasmática afectan negativamente a la congelación espermática.

**5.** Las concentraciones elevadas de prolactina plasmática afectan negativamente a la congelación espermática.

# BIBLIOGRAFÍA

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- Aboagla EME. Terada T. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology 2004; 62(6):1160–1172.
- Asher GW, Monfort SL, Wemmer C. Comparative reproductive function in cervids: implications for management of farm and zoo populations. Journal of Reproduction and Fertility 1999; 97:301–310.
- Axelrod J. The pineal gland: a neurochemical transducer. Science 1974; 28; 184(4144):1341–8.
- Bronson FH. Seasonal strategies: ultimate factors. Mammalian Reproductive Biology 1989; 28–59.
- Bóveda P, Toledano-Díaz A, Castaño C, Esteso MC, López-Sebastián A, Rizos D, Bielli A, Ungerfeld R, Santiago-Moreno J. Ultra-rapid cooling of ibex sperm by spheres method does not induce a vitreous extracellular state and increases the membrane damages. Plos One 2020; 15(1):e0227946.
- Brooks DE. Epididymal functions and their hormonal regulation. Australian Journal of Biological Science 1983; 36(3):205–222.
- Caldwell KB, Goff HD, Stanley DW. A Low-Temperature Scanning Electron Microscopy Study of Ice Cream. II. Influence of Selected Ingredients and Processes. Food Structure 1992; 11:11–23.
- Calzada L, Bernal A, Loustaunau E. Effect of steroid hormones and capacitation on membrane potential of human spermatozoa. Archives of Andrology 1988; 21:121–128.
- Campbell RC, Dott HM, Glover TD. Nigrosin eosin as a stain for differentiating live and dead spermatozoa. The Journal of Agricultural Science 1956; 48:1–8.
- Coloma MA, Gómez-Brunet A, Velázquez R, Toledano-Díaz A, López-Sebastián A, Santiago-Moreno J. Freezability of Iberian ibex (*Capra pyrenaica*) spermatozoa according to the glycerolization temperature and plasma testosterone concentration. Cryobiology 2010; 61(2):204–210.
- Coloma MA, Toledano-Díaz A, Castaño C, Velázquez R, Gómez-Brunet A, López-Sebastián A, Santiago-Moreno J. Seasonal variation in reproductive physiological status in the Iberian ibex (*Capra pyrenaica*) and its relationship with sperm freezability. Theriogenology 2011; 76:1695–1705.
- Cook KLM, Hartel RW. Ice Crystallization in Ice Cream Production. Comprehensive reviews in Food Science and Food Safety 2010; 9:213–222.
- Courtens JL, Paquignon M. Ultrastructure of fresh, frozen and frozen-thawed spermatozoa of the boar in: L.A. Johnson, K. Larsson (Eds.), Proceedings of the First International Conference on Deep Freezing of Boar Semen, Swedish University of Agricultural Sciences 1985; 61–87.

- Curry MR. Cryopreservation of semen from domestic livestock. Reviews of Reproduction 2000; 5(1):46-52.
- Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH. Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. Journal of Experimental Zoology 1993; 265(4):432–437.
- Eddy EM, O'Brien DA. The Physiology of Reproduction, In: In: Knobil, E., Neill, J. (Eds.) 1994; 1:29–77.
- Ekwall H. Cryo-scanning electron microscopy (Cryo-SEM) of boar semen frozen in medium-straws and MiniFlatPacks 2007; 67:1463–1472.
- Esteso MC, Fernández-Santos MR, Soler AJ, Garde JJ. Head dimensions of cryopreserved red deer spermatozoa are affected by thawing procedure. Cryo-Letters 2003; 24:261–268.
- Esteso MC, Soler AJ, Fernández-Santos MR, Quintero-Moreno AA, Garde JJ. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples. Journal of Andrology 2006; 27(5):662–670.
- Esteso MC, Rodríguez E, Toledano-Díaz A, Castaño C, Pradiee J, López-Sebastián A, et al. Descriptive analysis of sperm head morphometry in Iberian ibex (*Capra pyrenaica*): optimum sampling procedure and staining methods using Sperm-Class Analyzer®. Animal Reproduction Science 2015; 155:42–49.
- Flores-Gil VN, Velázquez R, Toledano-Díaz A, Santiago-Moreno J, López-Sebastián A. Short term administration of cyproterone acetate for contraception: effects on testosterone secretion and semen characteristics in rams (*Ovis aries*) and bucks (*Capra hircus*). Spanish Journal of Agricultural Research 2020; 18(4):e0407.
- Flores-Gil VN, Toledano-Díaz A, Velázquez R, Oteo M, López-Sebastián A, Santiago-Moreno J. Role of changes in plasma prolactin concentrations on ram and buck sperm cryoresistance. Domestic Animal Endocrinology 2021; 76:106624.
- Forero-González RA, Celeghini ECC, Raphael CF, Andrade AFC, Bressan FF, Arruda RP. Effects of bovine sperm cryopreservation using different freezing techniques and cryoprotective agents on plasma, acrosomal and mitochondrial membranes. Andrologia 2012; 44:154–159.
- França LR, Becker-Silva SC, Chiarini-Garcia H. The length of the cycle of seminiferous epithelium in goats (*Capra hircus*). Tissue Cell 1950; 31:274–280.
- Frank H. Microscopic examination of semen. Artificial Insemination in Livestock Breeding. Pathological Division. Bureau of Animal Industry, Agricultural Research Administration. Circular 567 U.S. Department of Agriculture 1950; 12–16.
- Fujimoto K, Yamamoto T, Kitano T, Abé S. Promotion of cathepsin L activity in new spermatogonial apoptosis induced by prolactin. FEBS Letters 2002; 521:43–46.

- Gao DY, Ashworth E, Watson PF, Kleinhans FW, Mazur P, Critser JK. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride, and sucrose on spermolysis. Biology of Reproduction 1993; 49(1):112–123.
- Garde JJ, Soler AJ, Cassinello J, Crespo C, Malo AF, Espeso G, Gomendio M, Roldan ERS. Sperm cryopreservation in three species of endangered gazelles (*Gazella cuvieri, G. dama mhorr, and G. dorcas neglecta*). Biology of Reproduction 2003; 69(2):602–611.
- Gerlach T, Aurich JE. Regulation of seasonal reproductive activity in the stallion, ram and hamster. Animal Reproduction Science 2000; 58(3-4):197–213.
- González-Fernández L, Morrell JM, Peña FJ and Macías-García B. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology 2012; 78:415–422.
- Gosch B, Fischer K. Seasonal changes of testis volume and sperm quality in adult fallow deer (*Dama dama*) and their relationship to antler cycle. Journal of Reproduction and Fertility 1989; 85:7–17.
- Hair WM, Gubbay O, Jabbour HN, Lincoln GA. Prolactin receptor expression in human testis and accessory tissues: localization and function. Molecular Human Reproduction 2002; 8:606–11.
- Heideman PD, Bronson FH. An endogenous circannual rhythm of reproduction in a tropical bat, *Anoura geoffroyi*, is not entrained by photoperiod. Biology of Reproduction 1994; 50:607–614.
- Hidalgo M, Rodriguez I, Dorado JM. The effect of cryopreservation on sperm head morphometry in Florida male goat related to sperm freezability. Animal Reproduction Science 2007; 100(1-2):61–72.
- Holt WV. Basic aspects of frozen storage of semen. Animal Reproduction Science 2000; 62(1-3): 3–22.
- Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reproductive Biomedicine Online 2003; 6(2):191–200.
- Isachenko V, Isachenko E, Katkov I, Montag M, Dessole S, Nawroth F, et al. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapour: effect on motility, DNA integrity and fertilization ability. Biology of Reproduction 2004; 71:1167–73.
- Isachenko E, Isachenko V, Weiss JM, Kreienberg R, Katkov II, Schulz M, et al., Acrosomal status and mitochondrial activity of human spermatozoa vitrified with sucrose. Reproduction 2008; 136:167–173.
- Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journals of Reproduction & Fertility 1984; 70:219–228.

- Jiménez-Rabadán P, Soler AJ, Ramón M, García-Álvarez O, Maroto-Morales A, Iniesta-Cuerda M, Fernández-Santos MR, Montoro V, Pérez-Guzmán MD, Garde JJ. Influence of semen collection method on sperm cryoresistance in small ruminants. Animal Reproduction Science 2016; 167:103–108.
- Jones R. Plasma membrane structure and remodelling during sperm maturation in the epididymis. Journal of Reproduction and Fertility 1998; 53:73–84.
- Karsch FJ, Bittman EL, Foster RL, Goodman RL, Legan SJ, Robinson JE. Neuroendocrine basis of seasonal reproduction. Recent Progress in Hormone Research 1984; 40:185–232.
- Lincoln GA, Davidson W. The relationship between sexual and aggressive behaviour, and pituitary and testicular activity during the seasonal sexual cycle of rams, and the influence of photoperiod. Reproduction 1977; 49(2):267–276.
- Lincoln GA, Short RV. Seasonal breeding: nature's contraceptive. Recent Progress in Hormone Research 1980; 36.
- Lincoln GA, Clarke IJ. Evidence that melatonin acts in the pituitary gland through a dopamine-independent mechanism to mediate effects of daylength on the secretion of prolactin in the ram. Journal of Neuroendocrinology 1995; 7(8):637–43.
- Lincoln GA. Reproductive seasonality and maturation throughout the complete life-cycle in the mouflon ram (*Ovis musimon*). Animal Reproduction Science 1998; 53:87-105.
- Marco I, Vinas L, Velarde R, Pastor J, Lavin S. The stress response to repeated capture in mouflon (*Ovis ammon*): physiological, haematological and biochemical parameters. Journal of Veterinary Medicine Series A 1998; 45(1-10):243–1253.
- Martínez-Fresneda L, Castaño C, Bóveda P, Tesfaye D, Schellander K, Santiago-Moreno J, García-Vázquez FA. Epididymal and ejaculated sperm differ on their response to the cryopreservation and capacitation processes in mouflon (*Ovis musimon*). Scientific reports 2019; 9(1):1–113.
- Martínez-Fresneda L, Sylvester M, Shakeri F, Bunes A, Del Pozo JC, García-Vázquez FA, ... y Santiago-Moreno J. Differential proteome between ejaculate and epididymal sperm represents a key factor for sperm freezability in wild small ruminants. Cryobiology 2021; 99: 64–177.
- Morris GJ. Rapidly cooled human sperm: no evidence of intracellular ice formation. Human Reproduction 2006; 21:2075–2083.
- Morris GJ, Faszer K, Green JE, Draper DE, Grout BWW, Fonseca F. Rapidly cooled horse spermatozoa: Loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. Theriogenology 2007; 68:804–812.
- Morris GJ, Acton E, Murray BJ, Fonseca F. Freezing injury: the special case of the sperm cell. Cryobiology 2012; 64(2):71–180.

- O'Brien E, Esteso MC, Castaño C, Toledano-Díaz A, Bóveda P, Martínez-Fresneda L, et al. Effectiveness of ultra-rapid cryopreservation of sperm from endangered species, examined by morphometric means. Theriogenology 2019; 129:160–167.
- Pintado B, Pérez-Llano B. Effect of glutaraldehyde concentration and fixative temperature on the number of spermatozoa with normal acrosomes in goat semen. Theriogenology 1992; 38:527–533.
- Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. International Journal of Andrology 2012; 35:109–124.
- Pradiee J, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, Santiago-Moreno J. Cryopreservation of epididymal sperm from ibexes *(Capra pyrenaica*) using short equilibration time with glycerol. Theriogenology 2014; 825:25–528.
- Pradiee J, Esteso MC, Lopez-Sebastián A, Toledano-Díaz A, Castaño C, Carrizosa JA, Urrutia B, Santiago-Moreno J. Successful ultra-rapid cryopreservation of wild Iberian ibex (*Capra pyrenaica*) spermatozoa. Theriogenology 2015; 84(9):1513–1522.
- Pradiee J, O'Brien E, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, et al., Effect of shortening the prefreezing equilibration time with glycerol on the quality of chamois (*Rupicapra pyrenaica*), ibex (*Capra pyrenaica*), mouflon (*Ovis musimon*) and aoudad (*Ammotragus lervia*) ejaculates. Animal Reproduction Science 2016; 171:121–128.
- Pradiee J, Esteso MC, Castaño C, Toledano-Díaz A, López-Sebastián A, Guerra R, et al., Conventional slow freezing cryopreserves mouflon spermatozoa better than vitrification. Andrologia 2017; 49:1–7.
- Pujianto DA, Curry BJ, Aitken RJ. Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. Endocrinology 2010; 151:1269–1279.
- Purohit SB, Saxena D, Laloraya M, Kumar GP. Altered molecular dynamics and antioxidant status in the spermatozoa in testosterone-induced oligospermia in mouse. Molecular Reproduction and Development: Incorporating Gamete Research 2000; 55(3): 316–325.
- Ramírez AR, Castro MA, Angulo C, Ramió L, Rivera MM, Torres M, ... Concha II. The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility. Biology of Reproduction 2009; 80(4):753–761.
- Rath D, Niemann H. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology 1997; 47:785–793.
- Reddy YD, Reddy KV, Govindappa S. Effect of Prolactin and Bromocriptine Administration on Epididymal Function - A Biochemical Study in Rats. Indian Journal of Physiology and Pharmacology 1985; 29:234–38.

- Sánchez R, Risopatrón J, Schulz M, Villegas J, Isachenko V, Kreinberg R, et al., Canine sperm vitrification with sucrose: effect on sperm function. Andrologia 2011; 43:233–241.
- Santiago-Moreno J, Gómez-Brunet A, González-Bulnes A, Toledano-Díaz A, Malpaux B, López-Sebastián A. Differences in reproductive pattern between wild and domestic rams are not associated with inter-specific annual variations in plasma prolactin and melatonin concentrations. Domestic Animal Endocrinology 2005; 28:416–429.
- Santiago-Moreno J, Toledano-Díaz A, Pulido-Pastor A, Dorado J, Gómez-Brunet A, López-Sebastián A. Effect of egg yolk concentration on cryopreserving Spanish ibex (*Capra pyrenaica*) epididymal spermatozoa. Theriogenology 2006a; 66:1219-1226.
- Santiago-Moreno J, Gómez A, Toledano A, González A, López-Sebastián A. Seasonal endocrine changes and breeding activity in Mediterranean wild ruminants. Reproduction in Domestic Animals 2006b; 41(2):72–81.
- Santiago-Moreno J, López-Sebastián A. Ungulados silvestres de España: biología y tecnologías reproductivas para su conservación y aprovechamiento cinegético. Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Ed.), Ministerio de Ciencia e Innovación, Monografías INIA: Serie Medioambiental, Madrid 2010.
- Santiago-Moreno J, Toledano-Díaz A, Sookhthezary A, Gómez-Guillamón F, Salas de la Vega R, Pulido-Pastor A, López-Sebastián A. Effects of anesthetic protocols on electroejaculation variables of Iberian ibex (*Capra pyrenaica*). Research in Veterinary Science 2011; 90:150-155.
- Santiago-Moreno J, Gómez-Brunet A, Toledano-Díaz A, Salas-Vega R, Gómez-Guillamón F, López-Sebastián A. Role of testosterone and photoperiod on seasonal changes in horn growth and sperm variables in the Iberian ibex, a model for polygynous wild bovids. Journal of Endocrinology 2012; 214:155–63.
- Santiago-Moreno J, Castaño C, Toledano-Dı´az A, Esteso MC, López-Sebastián A, Guerra R, et al. Cryopreservation of aoudad (*Ammotragus lervia sahariensis*) sperm obtained by transrectal ultrasound-guided massage of the accessory sex glands and electroejaculation. Theriogenology 2013; 79:383–391.
- Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: The Physiology of Reproduction, 2n Editions. Knobil E & Neill JD eds. Raven Press, Ltd, NY, USA 1994; 18:1063–1175.
- Shaw JM, Jones GM. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Human Reproduction Update 2003; 9:583–605.
- Sherman JK, Liu KC. Ultrastructure before freezing, while frozen, and after thawing in assessing cryoinjury of mouse epididymal spermatozoa. Cryobiology 1988; 19:503–510.

- Soler AJ, Esteso MC, Fernández-Santos MR, Garde JJ. Characteristics of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa cryopreserved after storage at 5 °C in the epididymis for several days. Theriogenology 2005; 64:1503–1517.
- Toledano-Díaz A, Santiago-Moreno J, Gómez-Brunet A, López-Sebastián A. Prolactin inhibition does not influence horn growth in two wild caprinae species: European mouflon (*Ovis orientalis musimon*) and Iberian ibex (*Capra pyrenaica*). European Journal of Wildlife Research 2012; 58(5):803–810.
- Varisli O, Uguz C, Agca C, Agca Y. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisosmotic solutions, cryoprotective agents and low temperatures. Animal Reproduction Science 2009; 110:256–268.
- Xu ZZ, McDonald MF, McCutcheon SN, Blair HT. Seasonal variation in testis size, gonadotrophin secretion and pituitary responsiveness to GnRH in rams of two breeds differing in time of onset of the breeding season. Animal Reproduction Science 1991; 26(3-4):281-292.

# ANEXO FOTOGRÁFICO

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**Figura 1.** Parques de machos monteses en condiciones de cautividad en el Departamento de Reproducción Animal del INIA (Madrid). A y B. Ejemplares animales en condiciones de cautividad con sus respectivos comederos.


**Figura 2.** Parques de muflones en condiciones de cautividad en el Departamento de Reproducción Animal del INIA (Madrid). A. Ejemplares en recintos de 250 $m^2$, con sus respectivos comederos. B. Ejemplares con su respectivo bebedero.


**Figura 3.** Manejo de ungulados silvestres. A. Manga de manejo. B. Capturadero. C. Inducción anestésica intravenosa dentro del capturadero.


**Figura 4.** Durante la inmovilización y el manejo optimización de condiciones para reducir el estrés. A. Muflón inmovilizado. B. Macho montés inmovilizado.


**Figura 5.** Monitorización y mantenimiento mediante anestesia inhalatoria en quirófano. A. Muflón monitorizado. B. Macho montés monitorizado.


**Figura 6.** Estudio ecográfico testicular. A. Realización de mediciones testiculares. B. Sonda ecográfica usada para la toma de medidas. C. Imágenes ecográficas observadas para la toma de medidas.

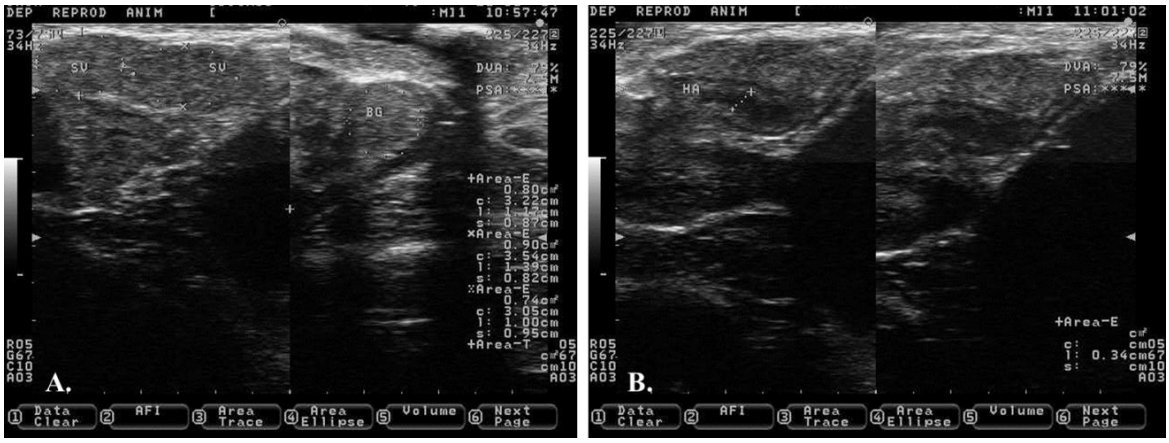


**Figura 7.** Estudio ecográfico de las glándulas sexuales accesorias. A. Vesículas seminales (SV, a la izquierda) y glándulas bulbouretrales (BG, a la derecha). B. Ampolla de Henle (HA).

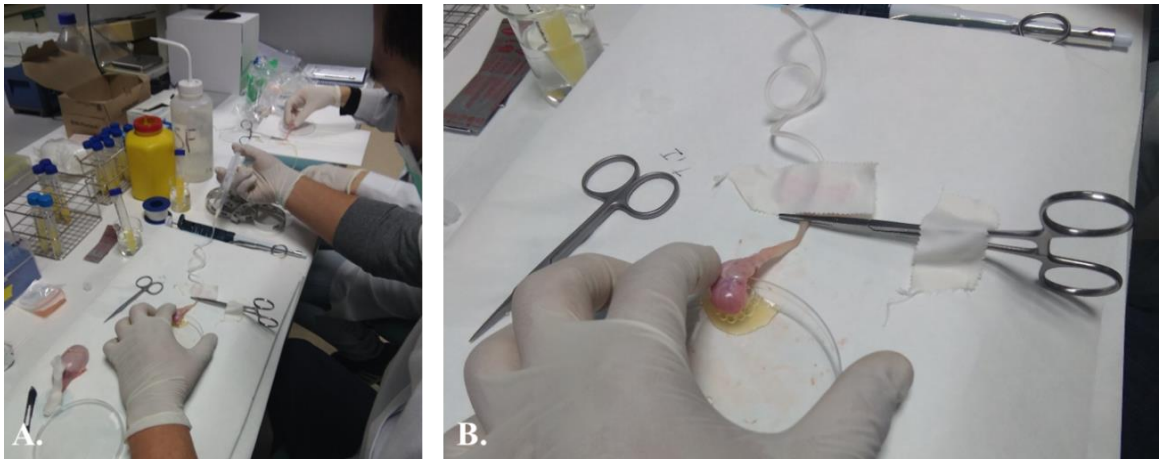


**Figura 8.** Recogida de muestras *post-mortem.* A. Flushing mediante lavado retrógrado a través del conducto deferente de la cola del epidídimo. B. Obtención de la muestras tras el flushing.


**Figura 9.** Método de recogida por masaje transrectal guiado por ultrasonido (TUMASG). A. Localización de las vesículas seminales. B. Pene exteriorizado con colector de vidrio estéril. C. Masaje transrectal. D. Estimulación eléctrica mediante electroeyaculador.

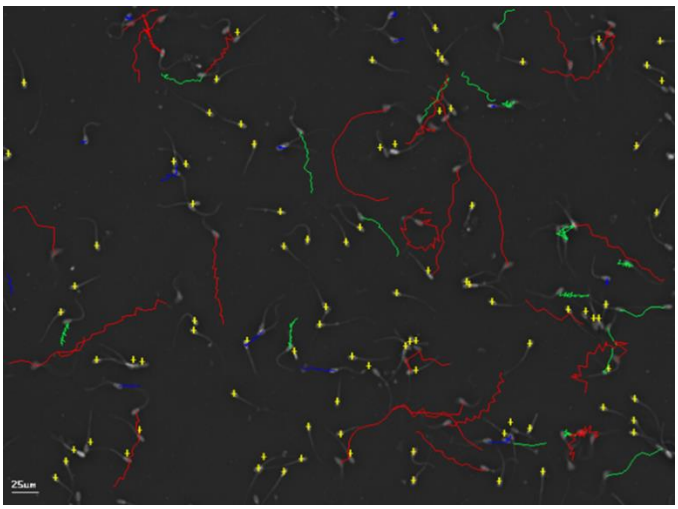

**Figura 10.** Captura con marcas cinéticas en espermatozoides, tomada mediante el sistema de análisis computerizado de imágenes (CASA; SCA).

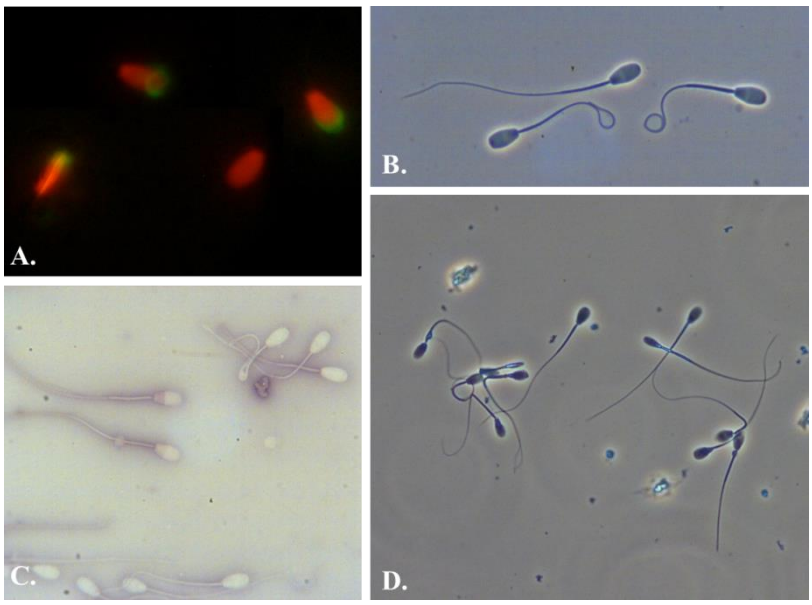


**Figura 11.** Diferentes parámetros espermáticos evaluados. A. Viabilidad celular e integridad del acrosoma mediante microscopía de fluorescencia. B. Test de integridad de membrana (HOST) mediante microscopía de contraste de fase. C. Tinción de viabilidad mediante eosina/nigrosina en microscopía de campo claro. D. Morfoanomalías en espermatozoides mediante microscopía de contraste de fases.

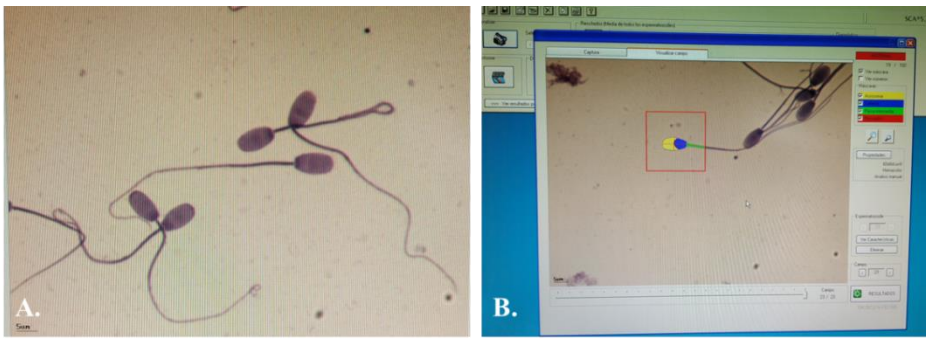


**Figura 12.** Análisis morfométrico de espermatozoides mediante el módulo de morfología dentro del SCA. A. Células espermáticas teñidas mediante la tinción hemacolor. B. Captura de cabeza espermática tomada por el programa.

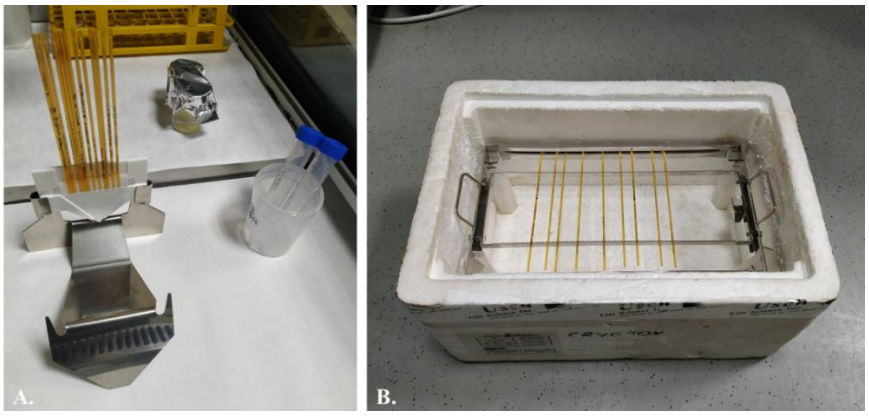


**Figura 13.** Método de congelación convencional. A. Refrigeración y empajuelado de las muestras espermáticas. B. Pajuelas situadas en el rack para la posterior congelación en vapores de nitrógeno.


**Figura 14.** Método de congelación ultra-rápida. A. Inmersión de muestras espermáticas en el nitrógeno líquido. B. Almacenamiento de pellets en criotubos. C. Aparato DPP70®.

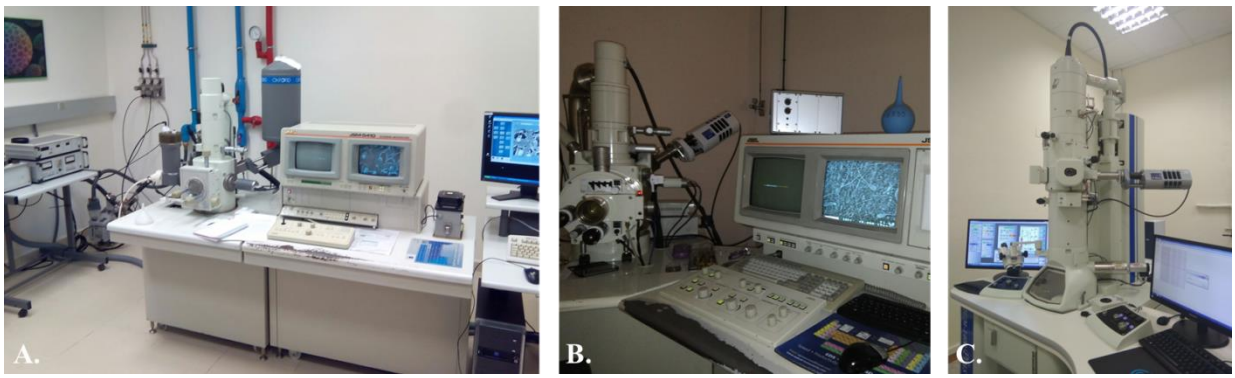


**Figura 15.** Técnicas de microscopía electrónica. A. Crio-microscopio electrónico de barrido (Crio-SEM). B. Microscopio electrónico de barrido (SEM). C. Microscopio electrónico de transmisión (TEM).